Oxidative stress on human proximal tubular cells in ischemia-reperfusion injury

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree Doctor of Medicine

by

Mr Avneesh Kumar

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DECLARATION

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any degree or other qualification.

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This research was carried out in the Institute of Ageing and Chronic Disease and Infection Immunology.

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Summary:

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Renal transplantation can often be complicated due to delayed graft function which is a direct sequela of ischemia reperfusion injury. Reactive oxygen species are the key mediators in this process causing direct cell damage which also initiate inflammation by inducing chemokines. The adverse outcome of delayed graft function is not only short term but long term function of the graft is also affected. The aim of this study was to measure the oxidative stress response in a cell culture system (immortalised human proximal renal tubular cells, HK-2) in response to a simulated pathological condition of ischemia reperfusion injury. The ability of these cells to produce chemokines in response to oxidative stress was also determined. Incubation with 0.5mM hydrogen peroxide produced a significant increase in the activity of the antioxidant enzyme glutathione peroxidase. Chemokines Interleukin-8 (IL-8; CXCL8) and MCP-1 (CCL2) were also induced after incubation with hydrogen peroxide. A dose related response was also observed. The cytokine Interleukin-1β (IL-1β) at 1ng/ml significantly potentiated the expression of both IL-8 (CXCL8) and MCP-1 (CCL2). Pre-incubation with an anti-oxidant (N-acetyl cysteine) strongly suppressed the induction of both IL-8 and MCP-1 when stimulated with hydrogen peroxide and IL-1β. In addition, enzymes concerned with detoxifying reactive oxygen intermediates were examined in renal transplant biopsies by Western blotting. Levels were

generally increased, though not significantly, in most biopsies, irrespective of diagnosis, but were also raised in pre-transplant kidney biopsies. This study identifies the role of oxidant injury in ischemia reperfusion injury and shows the underlying mechanisms of chemokine induction and suppression in oxidative stress. It also opens a new therapeutic window for anti-oxidants like N-acetyl cysteine in ameliorating this injury. These findings can have potential implications for clinical use to prevent ischemia reperfusion injury in renal transplantation.

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Abbreviations:

ACR	Acute cellular rejection
ADP	Adenosine diphoshate
ADPKD	Adult polycystic kidney disease
AP-1	Activator protein 1
AQP	Aquaporin
ATN	Acute tubular necrosis
ATP	Adenosine-5'-triphoshate
BCA	Bicinchoninic acid assay
CAN	Chronic Allograft Nephropathy
CAAT	Contigs-Assembly and Annotation Tool-Box
cAMP	Cyclic adenosine monophosphate
CGN	Chronic glomerulonephritis
CPN	Chronic pyelonephritis
CYA	Cyclosporine
DGF	Delayed graft function
DMSO	Dimethyl sulfoxide
DMTU	Dimethyl thiourea
DNA	Deoxyribonucleic acid
ECSOD	Extracellular Superoxide Dismutase
ET	Endothelin
GBM	Glomerular basement membrane
GPx	Glutathione Peroxidase
Grx	Glutaredoxin
GSH	Glutathione

HOCl	Hypochlorous acid
HUS	Haemolytic-uremic syndrome
IFN-γ	Interferon – gamma
IκB	Inhibitor of kappa B
IKK	IκB kinase
IL-1R1	Interleukin 1 receptor, type I
IRI	Ischemia-Reperfusion Injury
kDa	kilo Dalton
LPS	Lipopolysaccharide
MBL	Mannose-binding lectin
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MnSOD	Mitochondrial superoxide dismutase
mTAL	Medullary thick ascending limb
NAC	Acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NAL	Nacystelyn
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-kappa-B-inducing kinase
NMA	N-methyl-L-arginine
NO	Nitric oxide
$OONO^-$	Peroxynitrite
PBST	Phosphate buffered saline-+ 0.05% tween 20
PGI2	Prostacyclin
PMN	Polymorph nuclear leukocytes

PTC	Proximal Tubular Cells
PTLD	Post-transplant lymph proliferative disorder
ROS	Reactive oxygen species
RNI	Reactive nitrogen intermediates
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase's
TLR	Toll like receptor
TNF-α	Tumour necrosis factor – alpha
TxA2	Thromboxane A2
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TU	Thiourea
UW	University of Wisconsin

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

The kidneys are complicated organs that have numerous biological roles. The anatomical design of the kidney and its functional unit, the nephron, provides the primary route for the elimination of the end products of metabolism and of ingested foreign substances. Of equal or greater importance, however, is the role that the kidney plays in the regulation of extracellular fluid volume, osmolarity, pH and electrolyte composition.

Approximately one fifth of the cardiac output passes through the kidneys resulting in renal blood flow of up to 400ml per 100g of kidney per minute; a total of 170-180 L of plasma per day is filtered through the glomeruli at an approximate rate of 125ml/min (Raftery, 2000).

1.1 Anatomy of Kidney:

The kidneys are paired retroperitoneal organs situated on the posterior wall of the abdomen on each side of the vertebral column, at about the level of the twelfth rib. The left kidney is slightly higher in the abdomen than the right, due to the presence of the liver displacing the right kidney down. The normal kidney size of an adult human is about 12 x 6 x 3 cm with a weight of about 130gm (Mc-Minn, 1990). Cross section of a kidney shows a pale outer region, the cortex, and a darker inner region, the medulla (Figure 1).

The medulla is divided into 8-18 conical regions, called the renal pyramids; the base of each pyramid starts at the corticomedullary border, and the apex ends in the renal papilla which merges to form the renal pelvis and then on to form the ureter.

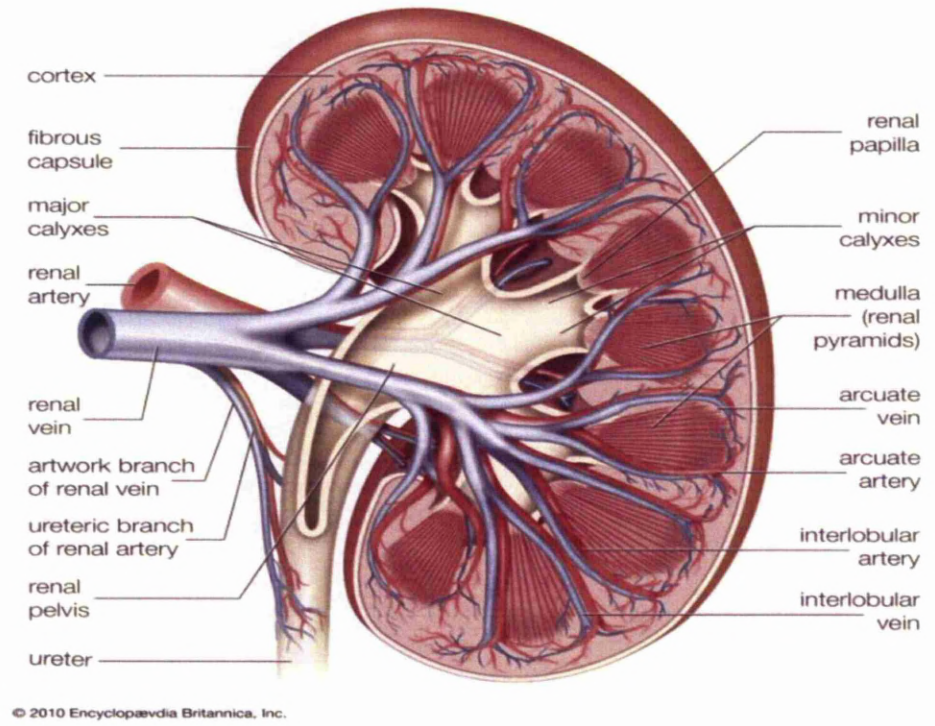


Figure 1.1: Cross section of the right kidney showing the major blood vessels.

(Human body: cross section of right kidney. Art. *Encyclopædia Britannica Online*)

1.2 Microscopic structure:

The nephron (Figure 2) is the basic functional unit of the kidney. Each kidney contains approximately 200,000 to over 1.8 million nephrons (Hoy et al, 2003). Each nephron is made up of glomerulus, proximal convoluted tubule, loop of Henle and distal convoluted tubule.

1.2.1 Glomerulus is made up of a tuft of capillaries which invaginates into the proximal blind end of the nephron called Bowman's capsule. The

epithelium covering the capillaries is continuous with that over the Bowman's capsule. The endothelium of the glomerulus is fenestrated with pores that are 70-90 nm in diameter and the epithelial cells have numerous pseudopodia that interdigitate to form filtration slits. The glomerular membrane acts as the main filtration mechanism and is impermeable to molecules larger than 4nm diameter (Ganong, 2001).

1.2.2 The proximal convoluted tubule is adjacent to Bowman's capsule and is responsible for controlled absorption of glucose and sodium, other solutes are also absorbed in this region.

1.2.3 The loop of Henle is the convoluted part of the proximal tubule which continues into the distal convoluted tubule. The thin descending limb is made of flattened cells whilst the ascending part is thick and made up of cuboidal cells. This region is responsible for concentration and dilution of urine by utilising a counter-current multiplying mechanism. It is water-impermeable but can pump sodium out, which in turn affects the osmolarity of the surrounding tissues and will affect the subsequent movement of water in or out of the water-permeable collecting duct.

1.2.4 The distal convoluted tubule is about 5mm long with few microvilli and has no distinct brush border. This region is responsible, along with the collecting duct that it joins, for absorbing water back into the body. 99% of the water is normally reabsorbed, leaving highly concentrated urine to flow into the collecting duct and then into the renal pelvis.

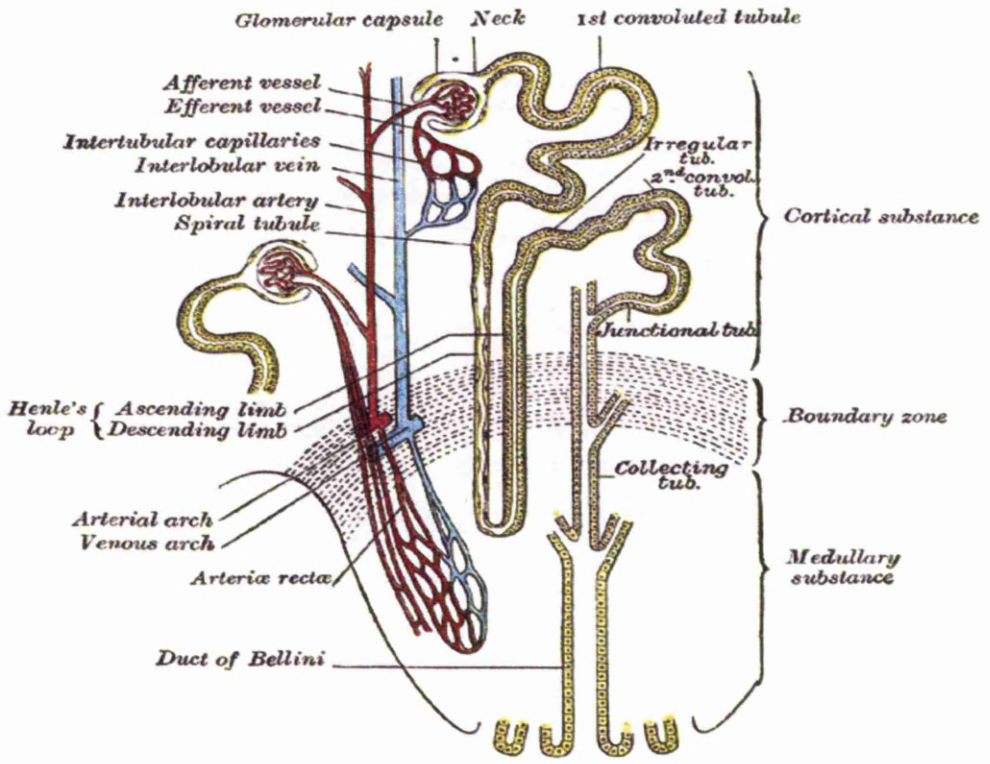


Figure 1.2: Nephron, the functional unit of kidney (*20th U.S. edition of* Gray's Anatomy of the Human Body)

1.3 Physiology:

The functions of the kidney can be divided into two groups: Endocrine function and extracellular homeostasis.

1.3.1 Endocrine function:

1. Secretion of erythropoietin, which regulates red blood cell production in the bone marrow.

2. Secretion of renin, which is a key part of the renin-angiotensin-aldosterone system.

3. Secretion of the active form of vitamin-D (Calcitriol) and prostaglandins.

1.3.2 Extracellular homeostasis:

The kidney's ability to perform many of its excretory functions depends on the three fundamental functions of filtration, re-absorption and secretion.

1.3.2.1 Filtration

The blood is filtered by nephrons, the functional units of the kidney. The glomerular filtration barrier, responsible for the size and charge-selective properties of renal filter, is composed of three separate layers: the fenestrated endothelium, the glomerular basement membrane (GBM), and the podocyte foot processes layer. Glomerular podocytes are highly differentiated cells with a complex cytoarchitecture. They have a voluminous cell body, long primary processes and regularly spaced, interdigitated foot processes that completely enwrap the glomerular capillaries (Gigante et al, 2011). The interdigitated foot processes of neighbouring podocytes cover the GBM and form a narrow filtration slit connected by an electron dense structure, called the slit diaphragm, a structure with a 40 nm diameter (Karnovsky and Ainsworth, 1972). The negative charge of the GBM was thought to provide a barrier to macromolecules (Kanwar, Linker and Farquhar, 1980) but instead a key role of podocytes has been described (Tryggvason, Patrakka and Wartiovaara, 2006), (Patrakka and Tryggvason, 2009). Cells, proteins, and other large molecules are filtered out of the glomerulus by a process of ultra filtration, leaving an ultra filtrate that resembles plasma (except that the ultra filtrate has negligible plasma proteins) to enter Bowman's space. Filtration is driven by Starling's force. The ultra filtrate is passed, in turn, through the proximal tubule, the loop of

Henle, the distal convoluted tubule, and a series of collecting ducts to form urine.

1.4 Proximal tubular cells (PTC):

The epithelial cells of the proximal tubule are also continuous with the epithelial cells of Bowman's capsule, but they differ in their morphology. The human PCT is about 15 mm long and 55 μm in diameter (Ganong, 2001). The cells lining the proximal tubule are cuboidal epithelial cells with deep basal membrane invaginations that provide a large basal surface area. The long microvilli (the brush border) lining the tubule lumen, maximize luminal surface area and make these cells ideally suited for both reabsorptive and secretory functions.

Furthermore the leaky nature of the apical tight junctions facilitates the para-cellular transport of water by osmosis, of solutes moving down electrochemical concentration gradients and of solutes moving by solvent drag.

Located in the luminal and basolateral membranes are enzymatic and protein carriers, primary and secondary active transport systems, which together with its permeability characteristics, make the proximal tubule the major site of re-absorption of the glomerular filtrate. It has been estimated that under normal circumstances 60 to 70 % of the glomerular filtrate is reabsorbed in the proximal tubule. The proximal tubule is further characterized by its leaky tight junctions and high permeability to water, by isosmotic reabsorption of filtrate under all conditions, by the relatively constant fraction of filtrate reabsorbed and by its being the site of origin of osmotic diuresis. The principal driving force for this large reabsorptive capacity is the primary active transport of Na^+

across the basolateral membrane of the epithelial cell. This mechanism is frequently referred to as the Na^+ pump. In the early proximal tubule this active movement of positive charge out of the tubule generates an electrical gradient which contributes to the driving force for the reabsorption of anions. The primary active transport of Na^+ across the basolateral membrane of the epithelial cell maintains a low intracellular Na^+ concentration relative to that in plasma and filtrate.

The Na^+ gradient across the luminal membrane provides energy for secondary active transport of a variety of filtered solutes. The re-absorption of solute establishes an osmotic gradient which, due to the high water permeability of the proximal tubule, drives the reabsorption of water at a rapid rate. The movement of water out of the tubule increases the luminal concentration of certain substances which are permeant but not actively transported, thus facilitating their passive re-absorption.

The proximal tubules (especially, the straight, distal portion or S3 segment) located in the outer medulla of the kidney are the primary site of injury in renal ischemia and reperfusion (Spiegel, Shanley and Molitoris, 1990). This region is marginally oxygenated under normal physiological conditions, with a high basal metabolic demand (Chen, Edwards and Layton, 2010; Whitehouse et al, 2006).

Therefore, with hypoxic or ischemic insult, proximal tubules in the outer medullary zone suffer the most damage. Such an ischemic injury, in the clinical world often leads to acute kidney injury. It also leads to delayed or non-functioning of transplanted kidneys.

1.5 Aquaporins: Aquaporins (AQP) are membrane water channels present in all life forms that play critical roles in controlling the water contents of cells (Borgnia et al, 1999). They permeate water, but block the transport of protons (Tajkhorshid et al, 2002). Eleven human AQPs have been identified, and their impaired function is implicated in pathological situations, such as nephrogenic diabetes insipidus (Borgnia et al, 1999).

2. Renal transplantation:

2.1 Introduction:

Renal transplantation offers patients with end stage renal failure improved survival and quality of life compared with dialysis. Not only it is the most physiological renal replacement therapy but it is also the most cost effective treatment. Although more transplants are being performed the size of the renal transplant waiting list is increasing at a faster rate. Data from the UK Renal Registry shows that since year 2000 there has been a 5% annual increase in prevalence of people requiring renal replacement therapy amounting to 2% of the total National Health Service budget (Galliford and Game, 2009). UK transplant is trying to increase the number of organ donations by improving the consent rate.

Overall long term graft survival and patient survival have remained unchanged since 1995. This lack of improvement has been shown, in spite of reduced early and late acute rejection rates (Meier-Kriesche et al, 2004) resulting in 5- and 10-yr graft survival rates of 70 and 50%, respectively (Gourishankar and Halloran, 2002).

The most common clinical correlate of late allograft loss is referred to non-specifically as interstitial fibrosis and tubular atrophy (IFTA), and more than

40% of kidney allograft recipients suffer from this during 5 yr after transplantation (Chapman, O'Connell and Nankivell, 2005).

IFTA is characterized by progressive renal allograft dysfunction with histopathological features of chronic interstitial fibrosis, tubular atrophy, vascular occlusive changes, and glomerulosclerosis. Thus, IFTA is a clinicopathological classification caused by multiple factors, including alloantigen-dependent immune responses, as well as nonimmunological factors, such as ischemia-reperfusion injury (IRI), calcineurin inhibitor nephrotoxicity, metabolic and cardiovascular disorders, and exacerbating pre-existing donor disease (Chapman, O'Connell and Nankivell, 2005).

2.11 Delayed graft function:

Delayed graft function (DGF) is a well-known complication affecting the kidney allograft in the immediate post-transplantation period. It is a form of acute renal failure resulting in post-transplantation oliguria, increased allograft immunogenicity and risk of acute rejection episodes, and decreased long-term survival. The frequency of DGF ranges from 5 to 50% in deceased-donor kidney transplants (Yarlagadda et al, 2009). DGF is usually the result of predominant ischemic injury to the graft before and during procurement and is further aggravated by the reperfusion syndrome. There are several risk factors that determine the incidence of DGF including: kidneys from non-heart-beating donor, inotropic support of the donor, cold storage preservation, cold ischemia time, donor age (>55 years), marginal kidneys from diabetic or hypertensive donors (Perico et al, 2004).

During the period of DGF the renal allograft remains quiescent and the patient requires dialysis support during this period. Patients require prolonged hospitalisation thus increasing the cost of transplantation and this also has an adverse effect on the rehabilitation of transplant recipients. Meta-analysis by Yarlagadda et al showed that patients with DGF had a 41% increased risk of graft loss and that patients with DGF had a higher mean serum creatinine. DGF was also associated with a 38% relative increase in the risk of acute rejection (Yarlagadda et al, 2009). Acute rejection of the allograft results from an increased expression of major histocompatibility complex (MHC) class I and II molecules in the kidney due to ischemic injury (Shoskes and Halloran, 1996). Several other authors have also a supported this view that DGF increases the incidence of acute rejection (McLaren et al, 1999), (Jushinskis et al, 2009).

Direct allorecognition is defined as the recognition by recipient T cells of intact MHC alloantigens displayed at the surface of donor dendritic cells carried within the graft. No other cells intervene in this initial step of the direct pathway (Land 2011).

In the indirect pathway of allorecognition, allogeneic MHC molecules and/or other donor alloantigen are processed and presented by recipient antigen presenting cells. The direct pathway plays a major role in the early weeks after transplantation and the indirect pathway may contribute to the process of chronic rejection (Hornick, 2006).

Apart from short term effects of DGF there is enough evidence accumulating in the literature about its deleterious effect on long term graft function and

survival (Giral-Classe et al, 1998), (Hassanain et al, 2009), (Yarlagadda et al, 2009).

Populations of renal cells after recovery from the initial injury might acquire a programmed tendency to proliferate, synthesise matrix, or otherwise contribute to chronic fibrosis. Inability of the kidney cells to regenerate completely, as seen in several animal studies of acute tubular necrosis, then will have reduced survival due to reduced nephron mass (Barrientos et al, 1994). These experimental results lend support to the finding that, in kidney-transplant recipients, delayed graft function is a risk factor for long-term renal allograft survival. Halloran et al have reported that the half-life of cadaveric kidneys with no delayed graft function was 11·5 years, compared with 7.2 years for those with delayed function (Halloran and Hunsicker, 2001).

The lengthening waiting list for renal transplantation has led to a relaxation in the selection criteria for kidney donors and the use of kidneys from so-called marginal donors. There is no widely accepted definition of what constitutes a marginal kidney, but examples include donors at the extremes of age, those with longstanding hypertension or diabetes, or donors where there is an increased risk of disease transmission. As might be expected, the results of transplantation with marginal kidneys are inferior to those with standard kidneys (Audard et al, 2008), (Ojo et al, 2001).

Kidneys from 'expanded criteria donors' and kidneys donated after cardiac death both are associated with a higher incidence of DGF and it is expected to become more clinically important in the near future. This makes a compelling case to investigate DGF further and to find novel solutions to ameliorate this phenomenon.

2.2 Oxidative stress:

Aerobic life depends upon controlled combustion for energy supply. Controlled combustion is catalyzed and regulated by metabolic machinery that can be damaged by uncontrolled oxidative reactions associated with energy production. Because of the extreme threat of such uncontrolled oxidation, aerobic life evolved a complex set of antioxidant systems to control these reactions and repair or replace the damaged machinery. At the same time, enzyme systems evolved to produce reactive species for biological signaling, biosynthetic reactions, chemical defence, and detoxification functions. The presence of both toxic and beneficial consequences of reactive species precludes a simple definition of oxidative stress (Jones, 2006). Oxidative stress was a term first described in 1985 by Helmut Sies (Sies, 1991) as "a disturbance in the pro-oxidant – antioxidant balance in favour of the former." However, it is important that the reactions in which free radicals are involved are not necessarily deleterious; on the contrary, they are of fundamental importance for life in that they take part in key biochemical reactions in all living organisms more useful contemporary definition is "a disruption of redox signaling and control" (Jones, 2006).

Reactive oxygen intermediates can influence the signaling of a wide variety of kinase pathways, such as the MAPK, NF-kB, CaMK and PI3K pathways (di Mari, Davis and Safirstein, 1999). These kinase pathways can have both anti-apoptotic and apoptotic roles (McCubrey and Franklin, 2006).

Neutrophil activation has been gaining attention in the past several years, primarily due to re-evaluation of the role of excessive inflammatory response in kidney ischemia reperfusion injury (IRI). In models of renal IRI, depletion of

neutrophils, blockade of neutrophil adhesion to the endothelium, and inhibition of the complement system all decrease kidney damage (Linfert, Chowdhry and Rabb, 2009). Recent studies have also indicated that ROS can participate in neutrophil recruitment by up-regulation of adhesion molecules and chemotactic factors.

Land et al have shown that such changes in chemokine and chemokine-receptor expression following transplantation regulate the migration of leukocytes from the peripheral circulation into an allograft (Land 2011).

Appropriate alloreactivity in vivo requires leukocyte interactions controlled within secondary lymphoid organs, and the migration of dendritic cells from the allograft into secondary lymphoid tissue is of paramount importance to the rejection process. In transplanted organs, ischemia /reperfusion, Toll like receptor (TLR) activation, cytokine stimulation and complement activation all induce expression of multiple pro-inflammatory chemokines in the grafts. These changes escalate IRI and amplify allograft rejection. Activation of TLR's on graft stromal cells initiates an inflammatory response characterized by the recruitment of cells to the sites of injury or infection by a tightly controlled sequence of events. This includes the expression of selectin, chemokine and chemokine-receptor genes that regulate cell migration to the sites of inflammation (Lutz, Thurmel and Heemann, 2010).

Key inflammatory chemokines induced include interleukin 8 (IL-8/CXCL8), growth-related oncogene (GRO/CXCL1), monocyte chemoattractant protein 1 (MCP-1/CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), macrophage inflammatory protein, (MIP-1α/CCL3, MIP-1β/CCL4) and RANTES (CCL5) (Zlotnik and Yoshie, 2000).

Oxidative stress is also shown to be increased in patients with chronic renal allograft nephropathy (CAN) (Raj et al, 2004).

During solid organ transplantation, both warm and cold ischemia are often unavoidable but can be minimized. To date, no specific therapy exists for the prevention or treatment of IRI. Treatment of IRI injury is still only supportive. Therefore, it is essential to develop preventive or therapeutic interventions and gain knowledge of the underlying mechanisms to prevent or reduce renal tissue injury following IRI.

2.3 Ischemia reperfusion injury:

Ischemia-reperfusion injury (IRI) is a common and important clinical problem in many different organ systems. It leads to tissue and organ damage after myocardial infarction, stroke, acute kidney injury, hepatic shock, mesenteric ischemia, and systemic shock. IRI is also common after deceased donor solid organ transplantation.

Cerra et al were among the first to describe reperfusion injury in 1975 in a canine model. They examined myocardial pedicles to detect the extent of reperfusion injury and found that increased ischemic times were associated with increased subendothelial hemorrhagic necrosis (Cerra et al, 1975).

Hemorrhagic shock leading to ischemic reperfusion injury has been shown to seriously damage the gut barrier, which included injury and atrophy in intestinal mucosa and increasing intestinal permeability (Chang et al, 2005).

IRI also affects the size and final outcome in cerebral and myocardial infarction (Boys et al, 2010).

IRI is a common problem that occurs when blood flow is interrupted to the kidney in case of kidney transplantation, aortic cross clamping and shock with subsequent resuscitation (Hussein et al, 2011). Hypothermic kidney storage before transplantation adds to ischemic tissue damage.

Ischemia-reperfusion injury is now recognized as a highly complex cascade of events that includes interactions between vascular endothelium, interstitial compartments, circulating cells, and numerous biochemical entities.

Inflammation is known to be a key mediator of IRI and considerable data exist demonstrating the significance of innate immunity (Boros and Bromberg, 2006), (Huang, Rabb and Womer, 2007).

The final stage of ischemic injury occurs during reperfusion. Reperfusion injury, the effector phase of ischemic injury, develops hours or days after the initial insult. Repair and regeneration processes occur together with cellular apoptosis, autophagy, and necrosis; the fate of the organ depends on whether cell death or regeneration prevails (Kosieradzki and Rowinski, 2008).

It is well established that a substantial component of post- ischemic injury is produced by oxygen-derived free radicals generated from xanthine oxidase at reperfusion. The absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation results in production of Reactive Oxygen Species (ROS). Oxidative damage from the ROS recruits polymorphs and lymphocytes at the site of injury and produces an inflammation like state. The polymorphs themselves increase the generation of ROS and also cause direct cellular injury rather than restoration of normal function.

Free radical-mediated injury releases proinflammatory cytokines and activates innate immunity. It has been suggested that all of these changes, the early innate response and the ischemic tissue damage, play roles in the development of adaptive responses, which in turn may lead to acute kidney rejection.

2.3.1 Pathophysiology of IRI:

Cellular hypoxia leads to depletion of energy and its substrates like Adenosine-5'-triphoshate (ATP) and Adenosine diphoshate (ADP). Lack of energy affects the function of the Na^+K^+ pump which is located at the cell membrane. Na^+K^+ pump via active transport of ions maintains higher intracellular concentration of potassium and lower concentration of sodium. The cell membrane becomes highly permeable leading to influx of Na^+ and Ca^{2+} into the cell and efflux of K^+ producing intracellular oedema.

2.3.1.1 Oedema: High-energy phosphates are vital for most cellular functions: from maintaining homeostasis, signal integration transduction, cell proliferation and differentiation to execution of the apoptotic death cycle. Although membrane phosphatases act slowly during ischemia, ATP depletion inhibits Na^+K^+ membrane phosphatase, thereby impairing the ability to maintain membrane potential and cell excitability, which require protection from gradient-driven K^+ and Na^+ ion trafficking. Sodium enters the cytoplasm accompanied by large amounts of water, producing oedema, the degree of which is dependent on the extent and duration of ischemia.

2.3.1.2 Calcium overload: Mitochondrial dysfunction is a critical event during ischemia, as it initiates both necrosis and apoptosis cascades during reperfusion. The organelle is both the site wherein ROS particles are produced and the preferred target of the injury. During ischemia, the Na^{+}/Ca^{2+} antiporter stops pumping calcium out of the cell, since sodium accumulating within the cell cannot be removed by the ineffective Na/K-ATPases, leading the Na/Ca exchanger to start to work in the reverse direction. Influx of extracellular calcium occurs only during prolonged ischemia and reperfusion (Kusuoka, Camilion de Hurtado and Marban, 1993).

Increased intracellular calcium activates calpain and causes translocation of Na/K ATP-hydrolase to the cytoplasm, increasing its deficit (Dunbar and Caplan, 2001). Mitochondrial permeability transition is a nonselective pore in the inner mitochondrial membrane, which is almost impermeable under physiological conditions, but opens due to a high mitochondrial content of calcium. Increased cytosolic calcium at neutral pH activates some cytoplasmic phospholipases and proteases. Two important groups of proteases that are activated in response to increased intracellular calcium are calpains (calcium-dependent cysteine proteases) and caspases. The former cleave a variety of proteins, including protein kinase C, fodrin, and cytoskeletal proteins while caspases have substrates within plasma membranes, lysosomes, mitochondria, and nucleus. Caspases are aspartate-specific cysteine proteases involved in the execution of apoptosis and cell death (Kosieradzki and Rowinski, 2008).

Free radicals are formed even during global ischemia, when small amounts of oxygen are available. Deamination of adenosine provides a substrate for xanthine oxidase. The enzyme xanthine dehydrogenase changes its conformation in response to ischemia. It is believed to result in free radical formation as shown in Figure 2.1. However, a more important source of free radicals in ischemia is the respiratory complex III itself (Chen et al, 2007).

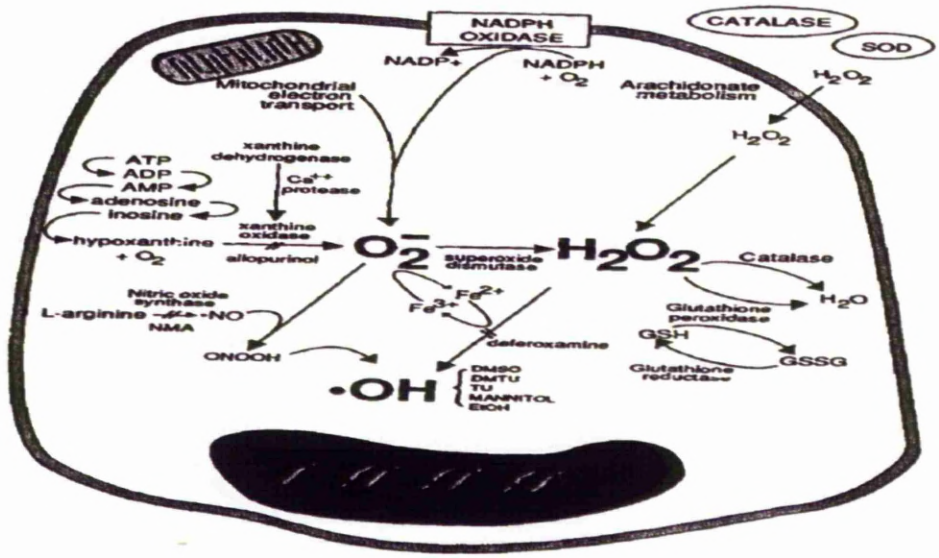


Figure 2.1: Potential intracellular pathways of ROS generation *SOD,* superoxide dismutase; *DMSO* dimethyl sulfoxide; *DMTU*, dimethyl thiourea; *TU*,thiourea; *NMA*, N methyl-L-arginine (DeForge et al, 1993)

2.4 Reperfusion Injury:

Reperfusion injury follows the ischaemic phase which occurs within hours and days of the initial insult. This period is highlighted by repair and regeneration. At cellular level apoptosis, autophagy and necrosis all occur together. Influx of calcium is well known to occur during the ischaemic phase due to disruption of Na/K pump but the levels return to normal and cells are able to make a recovery. An increase of cytoplasmic calcium which does not return to normal may be seen in irreversible injury. (Lee and Allen, 1992).

When the circulation is re-established oxygenated blood ushers a release of large amount of free radicles the source of which is likely from the respiratory chain. Mitochondria are the key players and regulators during this phase. The acidic environment during ischemia may be responsible for keeping the permeability pores closed but these open in reperfusion phase (Kim, Jin and Lemasters 2006). Free radicles cause direct damage to surrounding cell membranes by lipid peroxidation. Endothelial cells are important mediators of this injury which are well preserved during ischemic phase but during reperfusion they become oedematous.Reactive oxygen species (ROS) are known to stimulate production of key transcription factors, growth factors and releasing inflammatory mediators like Interleukin-8 (IL-8; CXCL8), Monocyte chemoattractant protein-1 (MCP-1; CCL2), and regulated on normal T cell expressed and secreted (RANTES; CCL5) (Lakshminarayanan et al, 1997). These chemokines recruit polymorphs and lymphocytes leading to cellular injury (Larsen et al, 1989). The final pathway leads to apoptosis and cell necrosis ending in destruction of tubules and fibrosis as shown in figure 2.2.

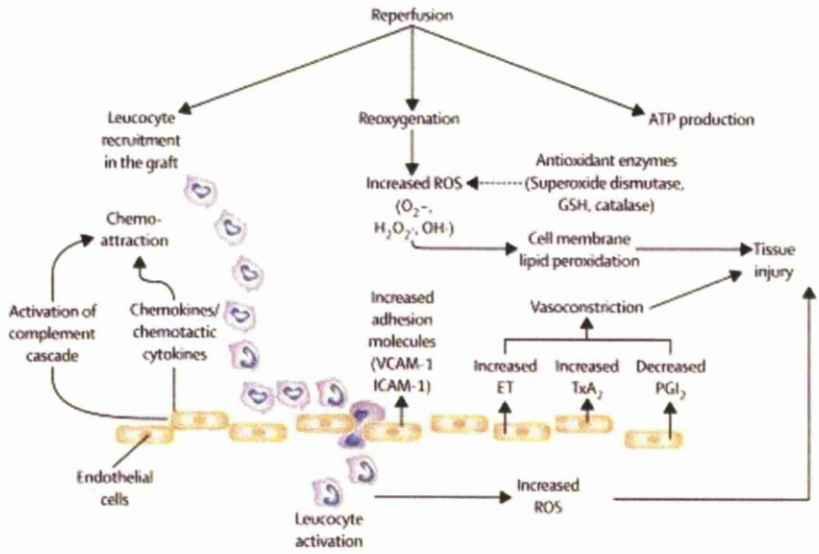


Figure 2.2: Schematic representation of leucocyte recruitment, endothelial cell activation, and generation of inflammatory and vasoactive mediators that perpetuate tissue injury after graft reperfusion *ROS*, reactive oxygen species; *GSH,* glutathione; *TxA2*, thromboxane A2; *ET*, endothelin; *PGI2*, prostacyclin; Dashed lines indicate inhibition (Perico et al, 2004).

The role of the complement system in IRI is long established. Increase in the production of complement components is regulated by pro-inflammatory cytokines: primarily IL-6, TNF-α and IFN-γ. While the importance of the alternative activation pathway has been demonstrated, recent evidence from mice suggests an additional role of the lectin-binding pathway. Ischemia and subsequent reperfusion leads to renal mannose-binding lectin (MBL) depositions in the early reperfusion phase, followed by deposition of C3, C6 and C9 in the later reperfusion phase. In the kidney, the deposition of MBL-A and MBL-C co-localized with C6, demonstrated that MBL is involved in complement activation. MBL-depositions in peritubular capillaries and tubular epithelial cells can also be observed early after transplantation of ischemically injured kidneys (de Vries et al, 2004).

Among the primary sources of ROS in kidney IRI are activated neutrophils, xanthine oxidase mediated conversion of xanthine to hypoxanthine, the mitochondrial electron transport chain, microsomal oxidation, and arachidonic acid metabolism. One of the major controversies regarding ROS generated during IRI is the mechanism of their involvement in the injury process. Although their harmful effects on lipids, proteins, and DNA are more or less understood, the ability of ROS and antioxidants to directly affect cellular signalling and in that way control gene expression needs additional investigation.

2.4.1 Reactive oxygen species:

Free radicals are small, diffusible molecules that have an unpaired electron. They tend to be reactive and can participate in chain reactions in which a single free radical initiation event can be propagated to damage multiple molecules. Polyunsaturated fatty acids, which are abundant in cell membranes, are oxidatively damaged by free radical chain reactions when exposed to O_2 in the presence of trace metal ions. This process is known as lipid peroxidation.

2.4.1.1 Mitochondrial ROS Generation

2.4.1.2 Superoxide (O_2^-)

The superoxide anion is created from molecular oxygen by the addition of an electron. It is not highly reactive. It lacks the ability to penetrate lipid

membranes and is therefore enclosed in the compartment where it was produced. The formation of superoxide takes place spontaneously and mitochondrial respiration is the major source with 0.2% of oxygen consumed being normally converted into superoxide in a quiescent state (St-Pierre et al, 2002).

Dismutation of superoxide, both spontaneous and catalyzed by the superoxide dismutases, provides a major source of hydrogen peroxide in cells (*reaction 1*)

Reaction 1

$$O_2^- \cdot + O_2^- \cdot + 2H^+ \rightarrow H_2O_2 + O_2$$

2.4.1.3 Hydrogen peroxide:

Hydrogen peroxide (H_2O_2) is not a free radical but is nonetheless highly important much because of its ability to penetrate biological membranes. It plays a radical forming role as an intermediate in the production of more reactive ROS molecules including HOCl (hypochlorous acid) by the action of myeloperoxidase, an enzyme present in the phagosomes of neutrophils.

The cytotoxicity of hydrogen peroxide primarily occurs through its ability to generate hydroxyl radical through metal-catalyzed reactions, such as the Fenton reaction (Reaction 2).

Reaction 2

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + \cdot OH + {}^- OH$$

An important function of H_2O_2 is carried out in its role as an intracellular signalling molecule. H_2O_2 once produced by the above mentioned mechanisms is removed by at least three antioxidant enzyme systems, namely catalases, glutathione peroxidases and peroxiredoxins.

2.4.1.4 Hydroxyl radical:

Due to its strong reactivity with biomolecules, hydroxyl radical (•OH) is probably capable of doing more damage to biological systems than any other ROS. The Haber-Weiss reaction generates •OH from H_2O_2 and superoxide (O_2^-). The reaction is very slow, but is catalyzed by iron. The first step of the catalytic cycle involves reduction of ferric ion to ferrous:

$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$

The second step is the Fenton reaction:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$

Net reaction:

$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + HO^- + O_2$

2.4.1.5 Nitric oxide:

Nitric oxide (NO) represents an odd member of the free radical family and is similar to O_2^- in several aspects in that it does not readily react with most biomolecules despite its unpaired electron. On the other hand it easily reacts with other free radicals (e.g. peroxyl and alkyl radicals), generating mainly less reactive molecules. O_2^- in parallel with NO react with each other to give $OONO^-$ (peroxynitrite), which is highly cytotoxic (Beckman and Koppenol, 1996).

Peroxynitrite (or its protonated form ONOOH) is a strong oxidizing agent and can lead to depletion of thiol groups, damage to DNA, and nitration of proteins.

$O_2^- \cdot + NO \cdot \rightarrow ONOO^-$

In physiological concentrations NO functions mainly as an intracellular messenger stimulating guanylate cyclase and protein kinases, thereby relaxing smooth muscle in blood vessels.

The major intracellular reactive oxygen species and their metabolism are shown in Table 2.1.

Table 2.1: Major intracellular ROS molecules and their metabolism (Nordberg and Arnér, 2001)

ROS molecule	Main sources	Enzymatic defense systems	Product(s)
Superoxide ($O_2^{\bullet -}$)	'Leakage' of electrons from the electron transport chain	Superoxide dismutase (SOD)	$H_2O_2 + O_2$
		Superoxide reductase (in some bacteria)	H_2O_2
	Activated phagocytes		
	Xanthine oxidase		
	Flavoenzymes		
Hydrogen peroxide (H_2O_2)	From $O_2^{\bullet -}$ via superoxide dismutase (SOD)	Glutathione peroxidase	H_2O + GSSG
		Catalases	$H_2O + O_2$
	NADPH-oxidase (neutrophils)	Peroxiredoxins (Prx)	H_2O
	Glucose oxidase		
	Xanthine oxidase		
Hydroxyl radical ($^{\bullet}OH$)	From $O_2^{\bullet -}$ and H_2O_2 via transition metals (Fe or Cu)		
Nitric oxide (NO)	Nitric oxide synthases	Glutathione/TrxR	GSNO

2.4.1.6: Hydrogen peroxide and NF-κB:

Nuclear factor kappa-light-chain enhancer of activated B cells (NF-Kb) is a protein complex that controls the transcription of DNA. It is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens (Brasier, 2006), (Gilmore, 2006).

NF-κB belongs to the category of "rapid-acting" primary transcription factors. Transcription factors that are present in cells in an inactive state and do not require new protein synthesis to be activated. This allows NF-κB to be a first responder to harmful cellular stimuli.

IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) is a cellular protein that functions to inhibit the NF-κB transcription factor. IκBα inhibits NF-κB by masking the nuclear localization signals of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm (Jacobs and Harrison, 1998). In addition, IκBα blocks the ability of NF-κB transcription factors to bind to DNA, which is required for NF-κB's proper functioning (Verma et al, 1995).

IκB kinase (IKK) enzyme complex is part of the upstream NF-κB signal transduction cascade. IKK specifically, phosphorylates the inhibitory IκBα protein. This phosphorylation results in the dissociation of IκBα from NF-κB and thereby activates NF-κB (Karin, 1999).

NF-κB is located in the cytosol complexed with the inhibitory protein IκBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IKK. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA. The DNA/NF-κB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, in turn, is translated into protein, which results in a change of cell function (Perkins, 2007).

H_2O_2 concentrations required to activate NF-κB are typically in the range 0.1 to 1 mM, which is much higher than the 5–15 μM range observed during

inflammation. Higher levels of oxidative exposure can turn a potential positive stimulus by H_2O_2 into an inhibitory effect (Test and Weiss, 1984), (Liu and Zweier, 2001).

In 1991, Schreck et al were the first to demonstrate that direct addition of H_2O_2 to the culture medium of a subclone of Jurkat cells could activate NF-κB (Schreck, Rieber and Baeuerle, 1991).

NF-κB activation by H_2O_2 is highly cell-type specific and involves quite different mechanisms (Li and Karin, 1999). In T cells, H_2O_2 induces a Syk-mediated tyr 42 or an IKK-induced ser 32 and 36 phosphorylation of IκBα, depending on the expression of the inositol phosphatase SHIP-1. In epithelial cells, H_2O_2 triggers IKK complex activation through protein kinase D activation as shown in Figure 2.3.

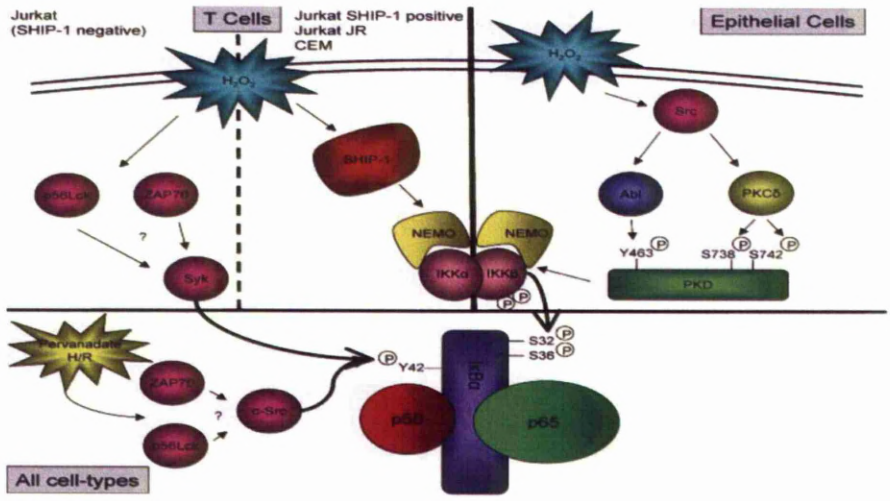


Figure 2.3: Mechanisms of H_2O_2-induced NF-κB activation in T cells and epithelial cells (Gloire, Legrand-Poels and Piette 2006)

NF-κB activation by Interleukin 1 receptor, type I (IL-1R1) depends on receptor endocytosis and endosomal ROS formation. IL-1β stimulation induces MyD88-dependent endocytosis of IL-1R1, an event required for NF-κB activation through Nox2-dependent H_2O_2 production as shown in Figure 2.4 (Gloire, Legrand-Poels and Piette, 2006).

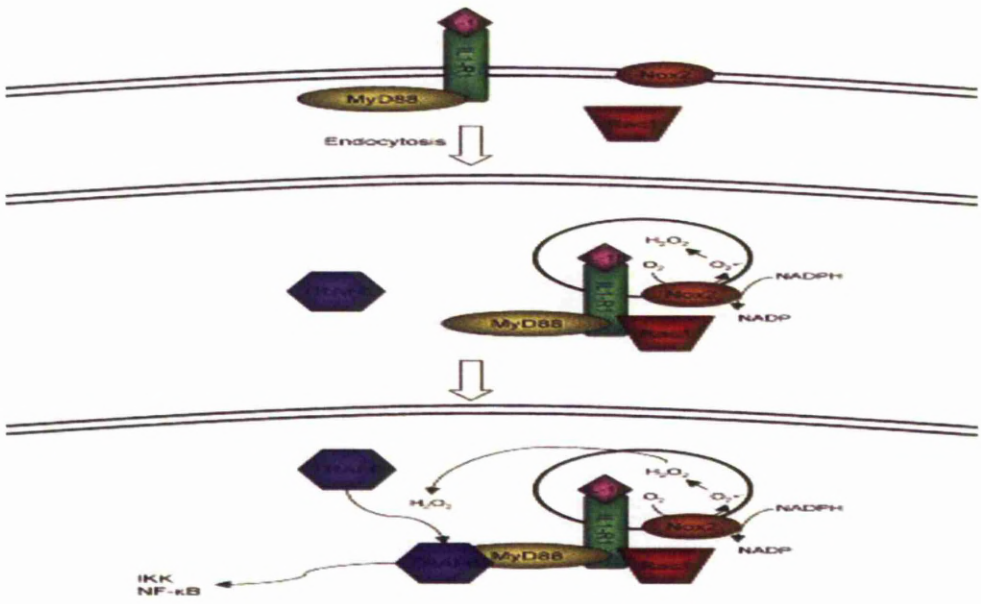


Figure 2.4: NF-κB activation by Interleukin 1 receptor, type I (IL-1R1) (Gloire, Legrand-Poels and Piette, 2006)

2.5 Antioxidants

Numerous antioxidant strategies exist and can be used to protect against ROS toxicity. The cellular antioxidant systems can be divided into two major groups, enzymatic and nonenzymatic. Major antioxidant enzyme systems are: superoxide dismutase, catalase, glutathione peroxidase (GPx), additional antioxidant enzymes such as peroxiredoxin, glutaredoxin (Grx),

and thioredoxin reductase (TrxR) also contribute to cellular protection against oxidation.

Manganese containing SOD (MnSOD) is localized in the mitochondria; copper and zinc containing SOD (CuZnSOD) is located in the cytoplasm and the nucleus; and extracellular SOD (ECSOD) is expressed extracellularly in some tissues. Other compartmentalized antioxidant enzymes include catalase, which is found in peroxisomes and the cytoplasm, and GPx, which can be found in many subcellular compartments including the mitochondria and the nucleus depending on the family member (Weydert and Cullen, 2010).

Intracellular enzymatic antioxidants are superoxide dismutase (SOD), catalase and GPx that convert potential substrates (superoxide anion radicals and hydrogen peroxide) to less reactive forms in the body (Halliwell and Gutteridge, 1995), (Rice-Evans and Burdon, 1993).

The major nonenzymatic antioxidant molecules are glutathione and vitamins A, C, and E (Finkel and Holbrook, 2000).

2.5.1 Superoxide dismutases (SOD)

Superoxide dismutases were the first genuine ROS-metabolizing enzymes discovered (McCord and Fridovich, 1969). They form the first line of defence against superoxide radicals as two isoenzymes exist, 80-kDa MnSOD present in mitochondria, and the cytosolic 32-kDa Cu/ZnSOD. In the reaction catalyzed by SOD, two molecules of superoxide form hydrogen peroxide and molecular oxygen and are thereby a source of cellular hydrogen peroxide.

$2O_2^{\bullet -}+2H^+ \rightarrow H_2O_2+O_2$

The reaction catalyzed by SOD is extremely efficient, limited in essence only by diffusion. In mitochondria, superoxide is formed in relatively high concentrations due to the leakage of electrons from the respiratory chain. MnSOD is essential or near essential to life since no inherited diseases have been found in which MnSOD is deficient, and knockout mice lacking MnSOD die soon after birth or suffer severe neurodegeneration (Melov et al, 1998). Expression of MnSOD is, in contrast to CuZnSOD, induced by oxidative stress and, interestingly and also by Thioredoxin (Das, Lewis-Molock and White, 1997).

Cytosolic CuZnSOD seems less important than MnSOD, and transgenic animals lacking this enzyme are able to adapt so that the phenotype appears normal (Ohlemiller et al, 1999).

2.5.2 Catalases

Catalases are mainly heme-containing enzymes. They are localised in mammalian cells in peroxisomes, where they catalyze the dismutation of hydrogen peroxide to water and molecular oxygen:

$2H_2O_2 \rightarrow O_2 + 2H_2O$

Another antioxidative role of catalase is to lower the risk of hydroxyl radical formation from H_2O_2 via the Fenton-reaction catalyzed by Copper or Iron ions (Halliwell, 1999).

Catalase binds to NADPH, which protects the enzyme from inactivation and increases its efficiency (Kirkman et al, 1999). Catalase activity assays typically involve the addition of its substrate (i.e H_2O_2), and the degradation of H_2O_2 is followed spectrophotometrically.

2.5.3 Glutathione peroxidases (GPx)

There are at least five different GPx in mammals (GPx1–5), all of which contain selenocysteine. GPx1 and GPx4 (or phospholipid hydroperoxide GPx) are both cytosolic enzymes abundant in most tissues (de Haan et al, 1998). GPx2 (gastrointestinal GPx) and GPx3 (plasma GPx) are mainly expressed in the gastrointestinal tract and kidney, respectively (de Haan et al, 1998), (Dreher et al, 1997). GPx3 may be catalytically regenerated by the Trx system (Bjornstedt et al, 1994). All glutathione peroxidases catalyze the reduction of H_2O_2 using glutathione as substrate.

$ROOH+2GSH \rightarrow ROH+GSSG+H_2O$

The catalytic mechanism proposed for reduction of hydroperoxides by GPx involves oxidation of the active site selenolate (Se^-) to selenenic acid (SeOH) (Epp, Ladenstein and Wendel, 1983).

Decreased GPx-1 activity can promote susceptibility to oxidative stress by allowing for the accumulation of harmful oxidants, whereas excess GPx-1 may promote reductive stress, characterized by a lack of essential ROS needed for cellular signalling processes. Excess oxidants or loss of essential ROS can each lead to diminished cell growth and promote apoptotic pathways (Lubos, Loscalzo and Handy, 2011).

2.5.4 Accessory antioxidant enzymes

Among these, the Thioredoxin (Trx), Glutaredoxin, and Peroxiredoxin systems are important contributors.

2.5.4.1 Thioredoxin system:

The thioredoxin system consists of the two antioxidant oxidoreductase enzymes Thioredoxin (Trx) and Thioredoxin reductase (TrxR). TrxR catalyzes the reduction of the active site disulphide in Trx using nicotinamide adenine dinucleotide phosphate (NADPH). Reduced Trx is a general protein disulfide reductant. Both TrxR and Trx have antioxidant properties (Nordberg and Arnér, 2001).

TrxR reduces the active site disulphide in thioredoxin and several other substrates directly under consumption of NADPH. Reduced thioredoxin is highly efficient in reducing disulphides in proteins and peptides, including peroxiredoxins and glutathione disulphide as shown in Figure 2.5.

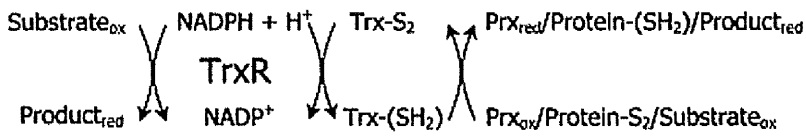


Figure 2.5: Enzymatic reactions of the thioredoxin system

Three distinct variants of human Trx have been characterized. The classical 12-kDa thioredoxin Trx-1, is known to be a multifunctional protein that can exist in the extracellular environment, cytoplasm and the nucleus (Arai et al, 2006). In the cytoplasm, Trx has been shown to regulate the redox environment of the cell and also the activity of certain proteins such as the apoptosis signal-regulating kinase-1 (Saitoh et al, 1998).

Trx-2 is located in mitochondria, mitochondrial Trx-2 is a 166 amino acid, 18 kDa protein with a conserved Trx catalytic site (Powis and Montfort, 2001).

Trx-2 has been shown to be critical in the regulation of mitochondrial membrane potential, NF-κB activation and apoptosis (Hansen, Zhang and Jones, 2006). Trx-2 seems to outweigh all the other mitochondrial defences in importance (Maulik and Das, 2008).

The third thioredoxin SpTrx, is a Trx expressed in spermatozoa (Miranda-Vizuete et al, 2001).

Trx modulates the signal transduction properties of ROS by reducing the intracellular disulphides induced by ROS and by lowering the levels of ROS directly. In the case of transcription factors, Trx enhance activator protein1(AP-1) activation. NF-κB activation, on the other hand, is clearly inhibited by reduced Trx in the cytosol (Kondo et al, 2006). Beneficial effects of thioredoxin, however may be a result of their capacity to act as a signalling molecule and not primarily as a radical scavenger. Recently, it was found that a combination of *N*-acetyl cysteine and thioredoxin might be far more effective than the administration of thioredoxin alone (Isowa et al, 2000).

2.5.4.2 Thioredoxin reductase:

Thioredoxin reductase (TrxR) contains selenium in the form of selenocysteine that catalyzes the NADPH-dependent reduction of thioredoxin, which is the major protein disulphide reductase in cells. They are involved in reduction of hydrogen peroxide and also regeneration of glutathione peroxidase (Matsui et al, 1996).

The molecular weight is 55kDa and in mammals, three isozymes of TrxRs have been identified: a cytosolic one (TrxR1) a mitochondrial one (TrxR2)

and a third isozyme highly expressed in testes, thioredoxin glutathione reductase (Bindoli et al, 2009).

Similar to TRX, glutaredoxin (GRX) is a thiodisulphide oxidoreductase that is involved in the protection and repair of protein and non-protein thiols during periods of oxidative stress (Berndt, Lillig, and Holmgren, 2007).

2.5.4.3 Glutathione:

Glutathione (GSH) is the most abundant intracellular thiol-based antioxidant, present in millimolar concentrations in all living aerobic cells. Its function is mainly as a sulphydryl buffer, but GSH also serves to detoxify compounds either via conjugation reactions catalyzed by glutathione S-transferases or directly. Oxidized glutathione is reduced by the NADPH-dependent flavoenzyme glutathione reductase. Oxidized glutathione may also be efficiently reduced by thioredoxins of E. coli, Plasmodium falciparum, and human origin. Hydrogen peroxide is reduced by glutathione peroxidases by oxidation of two molecules of glutathione forming glutathione disulphide that subsequently can be reduced by glutathione reductase under consumption of NADPH. Glutathione also reduces glutaredoxins (Grx) that in their turn reduce various substrates. Specific for glutaredoxins is the reduction of glutathione mixed disulphides such as glutathionylated proteins. Glutathione S-transferases catalyze the conjugation of glutathione with other molecules, thereby functioning as an intermediate step in the detoxification of miscellaneous toxic substances as shown in figure 2.6.

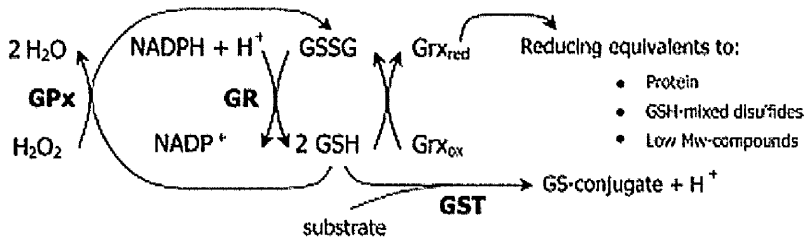


Figure 2.6: Schematic summary of the major glutathione-associated antioxidant systems (Nordberg and Arnér, 2001).

Several extracellular antioxidants such as proteins (transferrin, lactoferrin, albumin, cerruloplasmin) and urate prevent free radical reaction in the body sequestering transition metal ions by chelation in plasma. Albumin, bilirubin and urate may also scavenge free radicals directly. Furthermore, plasma has a considerable peroxyl radical scavenging ability, which is mainly determined by its content of ascorbic acid (Gutteridge, 1995), (Rice-Evans and Burdon, 1993), (Frei, England and Ames, 1989).

Some antioxidants are located both intra- and extra-cellularly, such as α-tocopherol, which is the major lipid-soluble antioxidant, present in cellular membranes and in plasma lipoproteins. It is an effective chain-breaking antioxidant that protects polyunsaturated lipids from peroxidation by scavenging peroxyl radicals (Halliwell, 1999).

2.5.4 N-acetyl cysteine:

Acetylcysteine (NAC) is the *N*-acetyl derivative of the amino acid L-cysteine, and is a precursor in the formation of the antioxidant glutathione in the body. The thiol (sulphydryl) group confers antioxidant effects and is able to reduce free radicals shown in figure 2.7.

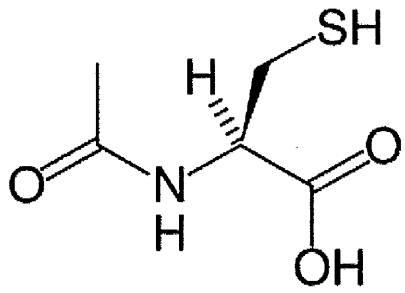


Figure 2.7: Chemical structure of N-acetyl cysteine

NAC is used clinically as a mucolytic drug and also in paracetamol over dosage. It has also been shown to prevent contrast induced nephropathy (Tepel et al, 2000). NAC is known as a drug that easily enters the cell and because of its -SH group, it is able not only to scavenge ROS, such as hydrogen peroxide and the hydroxyl radical, but it can also easily be deacetylated to cysteine, an important precursor of cellular glutathione synthesis. Therefore NAC scavenges oxidants both directly and indirectly. NAC has been tested in various laboratory models for its anti-oxidant properties. Ayvaz et al found that NAC administration caused an improvement in the histopathological findings of ischemia /reperfusion damages (Ayvaz et al, 2009). Fuller at al showed that donor pre-treatment in rats with NAC preserved renal metabolism and may improve outcomes of I/R injured kidney transplants (Fuller et al, 2004). Several rat models of IRI have also shown benefits of NAC (Di Giorno et al, 2006), (Sehirli et al, 2003).

NAC has also been shown to attenuate IRI in lung transplantation model (Inci et al, 2007) and to preserve myocardial function in acute myocardial infarction (Arstall et al, 1995). Other studies have also shown its role in inhibiting production of chemokines. Lappas et al (Lappas, Permezel and Rice, 2003) have shown that preincubation with NAC could suppress NFκB

activity and production of chemokines like IL-8. Wuyts have shown that N-acetylcysteine inhibits interleukin-17-induced interleukin-8 production from human airway smooth muscle cells (Wuyts et al, 2004).

2.6 Use of proximal tubular cells in cell culture:

There are many limitations in using in vivo models of renal IRI to elucidate the detailed signalling cascades. In vivo approaches must deal with multiple cell types within an organ and also with complex physiological control within an animal. In vitro studies with a pure population of a single cell type eliminate these complex external physiological influences and allow the direct study of signalling cascades. The proximal tubules (especially, the straight, distal portion or S3 segment) located in the outer medulla of the kidney are the primary site of injury in renal ischemia and reperfusion (Lieberthal and Nigam, 1998). This region is marginally oxygenated under normal physiological conditions, with a high basal metabolic demand. Therefore, with hypoxic or ischemic insult, proximal tubules in the outer medullary zone suffer the most damage.

Studies assessing mechanisms of proximal tubular cell physiology and pathophysiology increasingly utilize cell culture systems to avoid the complexity of whole organ/whole animal experiments. In vitro cell systems have proven very valuable in defining cell function and dysfunction in a variety of normal and disease states. In vitro cultures enable investigators to focus directly on the epithelium itself, removed from haemodynamic, neural, and hormonal factors that complicate studies of epithelia in vivo. A few investigators have reported the growth of human renal proximal tubular cell cultures in primary culture or early passage (Vesey et al, 2009).

However, ongoing harvesting of these cells is tedious and cell numbers from any given isolate are limited. Moreover, many investigators interested in human disease have no access to fresh human kidney tissue suitable for harvesting for in vitro studies (Racusen et al, 1997).

Research on IRI is often performed in animal models. Such in vivo methods may carry high risk of discomfort to the animal. Although animal models mimic physiological changes more closely, a cell culture model has several advantages:

- The cell population is well defined.
- The cells can be observed directly under a microscope.
- The physiological function of these cells is well maintained.
- There is easy access for biochemical (secretion, staining) analysis.
- The cells can also be stored and maintained for long periods.

Cell culture methods have been used in the past to study ischemia and reperfusion injury. Martou et al have used skeletal muscle cells (Martou et al, 2006), while Lee et al have used endothelial cells to simulate ischemia-reperfusion injury (Lee et al, 2009). Du et al have used human proximal tubular cells (HK-2) for a hypoxia / reoxygenation model of injury (Du et al, 2005). Daly et al have also used HK-2 cells to study effects of hypoxic pre-conditioning (Daly et al, 2009).

2.6.1 HK-2 cells:

Ryan et al described the first use of HK-2 cells for cell culture. Primary proximal tubular cell culture from normal adult human renal cortex exposed to a recombinant retrovirus containing the HPV 16 E6/E7 genes, resulted in a

cell line designated HK-2 (human kidney-2) which has grown continuously in serum free media for more than one year (Ryan et al, 1994).

HK-2 cell growth is epidermal growth factor dependent and the cells retain a phenotype indicative of well-differentiated proximal tubular cells (positive for alkaline phosphatase, gamma glutamyl transpeptidase, leucine aminopeptidase, acid phosphatase, cytokeratin, integrin, and fibronectin. Furthermore, HK-2 cells retain functional characteristics of proximal tubular epithelium (Na dependent l- phlorizin sensitive sugar transport; adenylate cyclase responsiveness to parathyroid hormone.

However, these changes do not equate with malignant transformation since the host cells retain features of differentiation, and thus, their growth is anchorage dependent and they remain under the control of many normal regulatory processes. Therefore, this type of cell preparation may represent a useful compromise between primary cultures and highly transformed cell lines. Many regulatory processes remain intact, and yet the need for repeated PTC isolation and characterization is eliminated (Ryan et al, 1994), (Racusen et al, 1997).

2.6.2 HK-2 Cells and Transport:

HK-2 cells have been used in transport studies of riboflavin (Kumar et al, 1998) and to study the function of the P-glycoprotein pump, multidrug resistance protein1, which is expressed in the basolateral basement membrane of HK-2 cells (Romiti, Tramonti and Chieli, 2002).

Studies of extracellular glutathione peroxidase have demonstrated polarization of HK-2 cells on permeable membranes. Extracellular glutathione peroxidase is secreted through the basolateral membrane of human kidney

proximal tubule cells into the extracellular fluid of the kidney, and from there enters blood plasma (Whitin et al, 2002).

2.7 Use of Hydrogen peroxide in cell culture:

Hydrogen peroxide has several advantages over superoxide as a cellular messenger. First, due to its uncharged nature it can diffuse more easily through membranes than can the superoxide anion. This might facilitate its removal by catalase within peroxisomes or by diffusion out of the cell. Hydrogen peroxide may also serve as a hormone-like extracellular stimulus; it is produced at sites of inflammation by neutrophils and macrophages, and could thus activate nearby cells. The second advantage may be its lower reactivity and toxicity.

Unlike superoxide, hydrogen peroxide is not a free radical. This increases its diffusion radius within the cytoplasm before decay. Finally, hydrogen peroxide can be completely detoxified in a single enzymatic step into H_2O and O_2 whereas superoxide is predominantly degraded via H_2O_2, and therefore causes prolonged toxicity.

Hydrogen peroxide is commonly used to study oxidative stress in proximal renal tubules (Andreucci et al, 2009), (Lee, Lee and Han, 2006), (Sheridan et al, 1996), (Yan et al, 2009).

2.8 Chemokines:

Chemokines are a family of chemotactic cytokines that were first identified on the basis of their ability to induce the migration of different cell types, particularly those of lymphoid origin. A wealth of data has demonstrated that chemokines working in concert with selectins and integrins act as directional signals to sort and direct effector leukocyte migration (Simonet et al, 1994),

(Baggiolini, Walz and Kunkel, 1989). In addition, chemokines have also been shown to activate leukocytes, influence haematopoiesis, and modulate angiogenesis (Li et al, 2005), (Singh et al, 2011). However, chemokines and their receptors are also important in dendritic cell maturation, B, and T cell development, Th1 and Th2 responses, infections, tumour growth as well as metastasis (Rossi and Zlotnik, 2000).

Like other cytokines, chemokines are secreted, and function locally in either paracrine or autocrine fashion. Furuichi and Daemen have shown chemokine involvement in mediating ischemic renal injury (Furuichi et al, 2008), (Daemen et al, 2001).

2.8.1 Members:

More than 50 human chemokines and 20 chemokine receptors have been described to date, with additional candidates currently under investigation (Ruffini et al, 2007). Chemokines can be classified into four subfamilies on the basis of the number and location of the cysteine residues at the N-terminus of the molecule and are named CXC, CC, CX_3C, and C, in agreement with the systematic nomenclature (Rollins, 1997).

2.8.2 Regulation of Chemokine Expression

Chemokines are regulated at transcriptional, post transcriptional, translational, and posttranslational levels (Holtmann et al, 1999). Many, but not all, of the proinflammatory chemokines are induced by IL-1β or tumour necrosis factor-α (TNF-α), RANTES and MCP-1 are upregulated in the presence of IL-2 (Loetscher et al, 1996), (Muselet-Charlier et al, 2007).

Some of the best studied pro-inflammatory chemokines (IL-8, RANTES, MCP-1) are controlled at the transcriptional level by the transcription factors

NF-kB, CAAT enhancer binding protein, and activator protein-1 (Hoffmann et al, 2002). Their activation requires a complex cascade of steps including phosphorylation by multiple kinases and phosphatases, degradation of transcriptional inhibitors, translocation of transcription factors from cytoplasm to nucleus. These signalling pathways can be different for each stimulus and transcription factor and are further complicated by "cross-talk" between the various pathways (Mukaida et al, 1994), (Oliveira et al, 1994).

Although at first glance this may appear to be an unnecessarily complicated system, it allows the fine-tuning and integration of the multiple signals required for a complex signal and tissue-specific biological response. These biochemical events are potential targets for therapeutic intervention. For example, glucocorticoids interfere with NF-kB activation (Bladh et al, 2005). Similarly, agents that influence cAMP levels often modulate the stimulatory signals for chemokine expression (Banerjee et al, 2011). The inhibition of chemokine expression seen after treatment with free radical scavengers may be due to interference with NF-kB activation, but may also involve other redox related steps (Haddad, 2002).

2.8.3 Principles of chemokine action:

Chemokines are produced by intrinsic cells as well as by infiltrating cells. They are released by stimulated cells and act locally. They act through G protein-coupled with transmembrane receptors and induce calcium influx. Chemokine receptor expression is cell-specific. They induce haptotaxis (cell migration along surface gradients) and also can activate adhesion molecules. Chemokines bind to proteoglycans and extracellular matrix, particularly on luminal surface of endothelial cells to attract leucocytes from blood into the

tissues. Activated cells have an increased production of ROS causing oxygen burst and the release of granules and proteinases by leukocytes. They can have a wide variety of actions and control aspects of immunomodulation, angiogenesis, hematopoiesis, and development as shown in figure 2.8.

The chemokine field has also received considerable attention since chemokine receptors were found to act as co-receptors for HIV infection (Alkhatib et al, 1996). In addition, chemokines, along with adhesion molecules, are crucial during inflammatory responses for a timely recruitment of specific leukocyte subpopulations to sites of tissue damage. Chemokines and their receptors are also important in dendritic cell maturation, B and T cell development, Th1 and Th2 responses, infections, angiogenesis, and tumour growth as well as metastasis (Vicari et al, 1997), (Strieter et al, 1995).

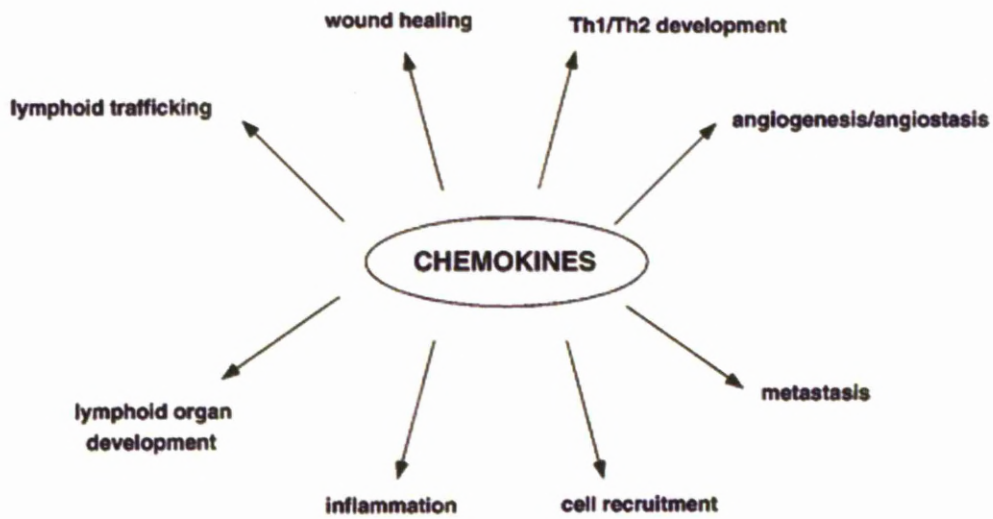


Figure 2.8: Biological functions of chemokines and chemokine receptors. (Rossi and Zlotnik, 2000)

Advances in understanding of chemokine receptor biology have highlighted that a controlled regulation of their activity is probably more important than their activation per se, certainly in the context of the immune system for both homeostasis and inflammatory responses. Individual receptors are subject to different mechanisms of regulation depending upon the type of cells on which they are expressed, the cell differentiation and activation status, as well as the microenvironment. Molecular mechanisms involved in the regulation are shared among chemokine receptors while others are purely receptor-specific, with either transient or permanent consequences on cell responsiveness to chemokine stimulation. The complexity of the regulation process confers specificity to what is an apparently redundant chemokine/chemokine receptor system (Bennett, Fox and Signoret, 2011).

2.8.4 IL-8:

Interleukin-8 (IL-8) represents a classical CXC chemokine and is a potent inducer of neutrophil chemotaxis (Yoshimura et al, 1987). It is produced early in inflammation as a direct result of interaction of cells with such stimuli as endotoxin, and it will also be further up-regulated by the early, alarm cytokines such as TNFα and IL-1. Reactive oxygen species, reactive nitrogen intermediates (RNI), and tissue injury are also frequent companions at sites of acute inflammation. The accepted hypothesis that links these observations may be stated as follows: PMN's are recruited to sites of acute inflammation; once at the site, they produce substantial quantities of ROS, which, together with released proteases injure the tissue. However, ROS/RNI may serve a completely different function in acute inflammation, where they do not

operate solely as end-stage effector molecules, but also as mediators regulating cytokine gene expression.

ROS have in fact been shown to be important inducers of both prokaryotic and eukaryotic gene expression by activation of various transcription factors including NF-κB (Ali and Sultana, 2011). The predicted sequence is as follows: low intracellular concentrations of ROS and RNI serve to up-regulate expression of IL-8, which then induces neutrophil chemotaxis. This hypothesis does not in any way contradict previous work concerning ROS / RNI and tissue damage, it merely expands their inflammatory role as initiators of the cascade that elicits neutrophils.

In contrast to other classic neutrophil chemoattractants such as leukotriene B4, C5, platelet activating factor, and the bacterial peptide f-MLP, IL-8 (CXCL8) is an extremely stable protein that is resistant to proteolysis and denaturation and has a prolonged biological activity in vivo. The stability of the IL-8 (CXCL8) protein and the sustained expression of its m-RNA suggest that the overall biological impact of IL-8 (CXCL8) may be greater than that of other neutrophil chemoattractants. Furthermore, since IL-8 (CXCL8) is produced de-novo in the presence of an inflammatory stimulus and not from pre-existing or latent factors, modulation of IL-8 (CXCL8) production may provide a more promising pharmacological target than other chemoattractants.

2.8.5 MCP-1

The monocyte chemoattractant protein-1 (MCP-1/CCL2) is a member of the C-C chemokine family, and a potent chemotactic factor for monocytes.

Human MCP-1 is composed of 76 amino acids and is 13 kDa in size (Van Coillie, Van Damme and Opdenakker, 1999).

MCP belongs to a family composed of at least four members (MCP-1, -2, -3, and -4). CCL2 is produced by a variety of cell types, either constitutively or after induction by oxidative stress, cytokines, or growth factors and is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages (Deshmane et al, 2009).

A biphasic expression of MCP-1(CCL2) and other chemokines, coinciding with the acute inflammatory and the later reparative phase, in renal ischemia reperfusion injury was observed by Stroo et al (Stroo et al, 2010).

Takaya et al have shown that in proximal tubules cells albumin was found to induce the transcription and translation of MCP-1 (CCL2) (Takaya et al, 2003).

2.8.6 RANTES

RANTES (for 'regulated upon activation normal T cell expressed and secreted') is a small protein of 68 amino acids which is also known as CCL5 (Zlotnik and Yoshie, 2000). The chemotactic activity of RANTES brings T cells, dendritic cells, eosinophils, NK cells, mast cells and basophils to sites of inflammation and infection. Whereas this cytokine was initially considered a T cell-specific protein, it has since been found to be produced by many cells in the body including platelets, macrophages, eosinophils, and fibroblasts, as well as endothelial, epithelial, and endometrial cells (Appay and Rowland-Jones, 2001).

2.9 AIMS OF THE STUDY:

The data from experimental animals and cell cultures have indicated that the formation of ROS and consequent oxidative stress play an important role in the pathogenesis of ischemia reperfusion injury. Not only do they cause direct cellular damage but they also induce chemokines like IL-8 (CXCL8) and MCP-1 (CCL2) which recruit neutrophils and monocyte respectively. This leads to an inflammatory state causing further damage to the affected organ. Antioxidant enzymes form the primary defence system against the ROS during reperfusion injury and this study was designed to evaluate the expression of different oxidant and antioxidant mechanisms in renal ischemia reperfusion injury.

The specific aims of the study were:

1. To measure antioxidant enzyme response in immortalised human proximal renal tubular cells after stimulation with hydrogenperoxide, a simulated pathological condition of ischemia reperfusion injury.
2. To determine the ability of proximal human tubular cells to produce chemokines like IL-8, MCP-1 and RANTES after hydrogenperoxide stimulation.
3. To determine if antioxidant like acetyl cysteine can suppress this stimulation.
4. To measure oxidative stress markers in kidney biopsies from renal transplant recipients and identify the role of oxidative stress in various pathological conditions and link it with the labarotary model.

CHAPTER 2

MATERIAL AND METHODS

3. METHODS

(A) Laboratory:

Measurement of markers of oxidative stress and adaptive responses in response to simulated pathological condition of ischemia reperfusion injury and to measure the induction of pro-inflammatory chemokines IL-8(CXCL8), MCP-1(CCL2) and RANTES(CCL5).

(B) Clinical:

Measurement of markers of oxidative stress from renal transplant recipients with IRI, acute cellular rejection (ACR) and chronic allograft nephropathy (CAN) from renal biopsy samples.

This project will attempt to link novel mechanisms of injury in cell culture experiments and oxidative stress responses in a clinical setting that occur during ischemia reperfusion injury, acute cellular rejection and chronic allograft nephropathy.

3.1 LABORATORY STUDIES

3.1.1 Materials:

HK-2 human kidney, proximal tubule cells, transformed, American Type Culture Collection (ATCC, CRL-2190) Rockville, MD

Keratinocyte Serum Free Media (KSFM, Invitrogen Lifetechnologies, 17005-042)

Fetal Bovine Serum, 100 ml, heat inactivated (Invitrogen Life technologies, 10082139)

Trypsin-EDTA (0.25% Trypsin, 1mM EDTA.4Na) (Invitrogen Life technologies, 25200)

Glutamine

HEPES buffer

Epidermal Growth Factor

Human CCL2/MCP-1 Immunoassay (Quantikine) DCPOO, R&D Systems

Human CXCL8/IL-8 Immunoassay (Quantikine) DY208, R&D Systems

Interleukin 1 beta (IL-1β) Human recombinant: T9401 (Sigma)

N- Acetyl cysteine: A9165 (Sigma)

Hydrogen peroxide 30% w/w soln: H1009 (Sigma)

Corning Brand Culture Flasks, T25 (Fisher Scientific, 10-126-28)

Corning Brand Culture Flasks, T75 (Fisher Scientific, 10-126-37)

Falcon polystyrene 35 mm dishes (Fisher Scientific, 08-722A)

Wheaton Cryule Vial (Cryovials) (Fisher Scientific, 03-341-18N)

Nalgene Cryogenic Controlled-Rate Freezing Container (Fisher Scientific, 15-350-50)

3.1.2 Equipment:

IEC PR-7000M centrifuge, rotor 966, International Equipment Company

Steril®GARD II Class II Type A/B3 Biosafety Cabinet, The Baker Company

Nuaire US Autoflow CO_2 water-jacketed incubator

Zeiss Axiovert25 phase contrast microscope

3.1.3 Cell Culture Techniques:

Immortalized human proximal renal tubular epithelial HK-2 cells were obtained from ATCC. Cells were tested and shown to be negative for mycoplasma using the Hoechst 33258 (Bisebenzamide) DNA staining

method (Sigma, Poole, UK). Cells were cultured until 90–95% confluent in six-well plates or T75 flasks in DMEM/Ham's F12 medium supplemented with 5.5 mM glucose, 2 mM L-glutamine, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 0.4 µg/ml hydrocortisone, 5 ng/ml epidermal growth factor, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 mM HEPES, and 10% FCS. These cells were used for no more than 10 passages. Media was changed on alternate days. All cells were grown in the appropriate growth media until they were about 80-90% confluent. HK-2 cells cultured with serum were growth arrested for 48h SFM. Cells cultured under serum free conditions were also given a 24h equilibration period after reaching the desired confluency.

3.1.3.1 Subculture and Feeding:

All conditions were under Biosafety Level 2 containment, performed in a Class II Biosafety Hood using sterile pipettes and plastic/glassware, wearing a laboratory coat and double-gloved. Subculture was performed weekly or when the monolayers reached 80% confluence as assessed by phase-contrast microscopy. Media from flask-grown cells were poured into a waste flask. The monolayers were rinsed with 1.5 ml/T25, 3 ml/T75 HEPES Buffered Saline Solution (HBSS) then trypsinized for 10 min to lift cells from flasks. Trypsinization was performed by adding 1.5ml /T25 or 3 ml/T75 0.25% trypsin, 0.05% EDTA (Invitrogen), decanting after 30 seconds and incubating the monolayer in the residual trypsin solution. When cells were released from the flasks (10 min), culture medium was added to each flask (5 ml/T25, 8 ml/T75) and swirled over the cells. The cell suspension was transferred to a 15 ml or 50 ml centrifuge tube and centrifuged at 270 x g for 5 min at 4°C

(IEC PR-7000M centrifuge, rotor 966). The supernatant was discarded and the pellet resuspended in culture medium. Using a hemocytometer, the cell suspension was enumerated and the cells seeded into new flasks or plates. Cells grown in T75 flasks were seeded at a density of $4.0x10^5$ cells in 25 ml or split at a one to four ratio and fed twice weekly. Cells grown in T25 flasks were seeded at a density of 1 to $1.5x10^6$ cells in 12 ml or at a one to four ratio and were fed every other day. Millicell inserts were seeded at $4.0x10^5$ cells in 2.0 ml culture medium in the basolateral compartment with 1.5 ml culture medium added to the apical compartment. Cells were fed every other day. Transwells were seeded at $4.0x10^5$ cells or superseeded at $1.41x10^6$ cells in 2.6 ml culture medium in the basolateral compartment with 1.5 ml culture medium added to the apical compartment. Cells were fed every other day or daily. Cells were maintained in an incubator at 37°C and 5% CO_2.

3.1.3.2 Cryopreservation of Cell Line:

Cells from intermittent passages were cryopreserved in a solution of culture medium (DMEM), 20% FBS and 10% dimethyl sulphoxide (DMSO) at a cell suspension of 1-1.5 x 10^6 cells/ml. The cryosuspension was prepared as a double-strength cryomedium (0.35 ml culture medium, 0.10 ml FBS, 0.05 ml DMSO per vial) to which a cell suspension (0.5 ml x 2.0 to $3.0x10^5$ cells/ml culture medium) was added dropwise to the cryovial for a final volume of 1ml cryosuspension. The cryovials were placed in a Nalgene cryofreezer temperature control unit and placed in a -20°C freezer overnight, transferred

to a - 80°C freezer for 4 hours then placed in liquid nitrogen or in a -135°C freezer for long-term storage.

3.1.3.3 Cell Pellets for Assays:

Cells grown in 35 mm plates, T25 or T75 flasks were rinsed X 2 with 1-3 ml HBSS, then scraped with a Teflon scraper, transferred into 15 ml or 50 ml centrifuge tubes and centrifuged at 270 x g for 5 min at 4°C (IEC PR-7000M centrifuge, rotor 966). Supernatant was discarded. Cells were stored at -80°C if not used immediately.

3.1.4 BIOCHEMICAL ANALYSIS

Cells were harvested in 550µl of PBS/well and sonicated for 10sec at 15 microns (Soniprep 150, Sanyo Scientific; n=6). The cell extracts were centrifuged, and the protein content was measured by the BCA method.

Catalase activity was measured using the method of Claiborne, based on the breakdown of H_2O_2 (Clairborne 1985).

Glutathione peroxidase (GPx) activity was measured using the method of Flohe and Gunzler (Flohe and Gunzler, 1984).

3.1.4.1 Analysis of protein content of samples:

Protein content of samples was measured by the bicinchoninic acid (BCA) method.

3.1.4.2 Bicinchoninic acid (BCA) method for determination of protein content:

Where specified the protein content of samples was determined using the bicinchoninc acid (BCA) protein assay kit (Sigma.) This method is based on the method developed by Smith (Smith et al, 1985).

Reagents

- Reagent A: Bicinchoninic acid (BCA) solution, containing: 25mM BCA-Na, 160mM $NaCO_3.H_2O$, 7.0mM Na_2 tartrate, 0.1mM NaOH and 0.95% $NaHCO_3$, pH 11.2 (Sigma-Aldrich, Dorset, U.K.)
- Reagent B: 160mM $CuSO_4.5H_2O$ (Sigma-Aldrich, Dorset, U.K.)
- Bovine serum albumin (BSA) 1mg/ml (Sigma-Aldrich, Dorset, U.K

Protocol

A range of standards from 25-250µg/ml were prepared from a stock solution of 1mg/ml bovine serum albumin (BSA). Twenty microlitres of sample or standard were placed in a 96 well microplate. Twenty microlitres of dH_2O was used as a blank. Reagent C was prepared by adding 500µl of Reagent B to 25ml of Reagent A. Two hundred microlitres of Reagent C was added to the samples, standards and blank, the microplate was incubated at 50°C for 30mins. The absorbance of samples and standards was then measured at 570nm using a microplate reader (Powerwave X340, Bio-tech instruments Inc, Vermont, USA.). The protein content of the samples was calculated using the standard curve.

3.1.4.3 Catalase assay:

Catalase activity was measured spectrophotometricaly using the assay developed by

Beers and Sizer (Beers and Sizer, 1952), and used by Claiborne. Catalase catalyses the decomposition of hydrogen peroxide to water and molecular oxygen. Catalase activity can be measured by the decomposition of hydrogen peroxide at 240nm.

Reagents

30% hydrogen peroxide (Sigma-Aldrich, Dorset, U.K)

50mM potassium phosphate buffer, pH 7.0

Protocol

The concentration of the stock 30% hydrogen peroxide was measured on each day the assay was performed as it degrades slowly on storage at 4°C. The stock hydrogen peroxide was diluted 1 in 800 with dH_2O and the absorbance at 240nm was measured (CECIL CE594/ Double Beam Spectrophotometer, Cambridge, U.K.) against a quartz cuvette containing distilled water. The concentration of the stock hydrogen peroxide was calculated using the molar extinction coefficient of $43.6M^{-1}cm^{1}$ (Claiborne, 1985). A 19mM hydrogen peroxide solution was prepared in 50mM phosphate buffer. Four hundred microlitres of this solution was placed in a quartz cuvette, twenty microlitres of sample was added to this and rapidly mixed. The decrease in absorbance at 240nm was monitored at room temperature over 1-2 minutes. The activity of catalase was determined as micromoles of hydrogen peroxide converted per milligram of protein in the

sample. The conversion of maximum velocity to specific activity of catalase is made as follows:

$$\text{Specific Activity (units/mg)} = \frac{\Delta A_{240nm} \text{ per minute x } 1000}{43.6 \text{ x } \frac{\text{mg protein}}{\text{ml reaction mix}}}$$

3.1.4.4 Glutathione peroxidase assay:

Glutathione peroxidase was measured spectrophotometrically using a method described by Flohe and Gunzler (Flohe and Gunzler, 1984).

Reagents

50mM Tris/HCl pH 7.6

Coupling reagent in 100ml Tris/HCl buffer:

2mM disodium EDTA, 1mM NaN_3, 1mM Glutathione (reduced form), 0.2mM NADPH, 100 Units glutathione reductase (600U/ml Roche Diagnostics Ltd,East Sussex, U.K.)

30% hydrogen peroxide (Sigma-Aldrich, Dorset, U.K.)

Protocol

The concentration of the stock 30% hydrogen peroxide was measured at 240nm as described for the catalase assay (section 3.1.4.3). A 1mM hydrogen peroxide solution was prepared in 50mM Tris/HCl buffer. Ten microlitres of sample was added to 575µl of coupling reagent in a quartz cuvette, 15µl of hydrogen peroxide was added to this and rapidly mixed. The decrease in absorbance at 340nm was measured for 1min (CECIL CE594/ Double Beam Spectrophotometer, Cambridge, U.K.). The decrease in absorbance of 585µl of coupling reagent with 15µl was used as a blank and

subtracted from that of the samples. The activity of glutathione peroxidase was calculated using the molar extinction coefficient of 6220 $M^{-1}cm^{1}$.

3.2 MODEL FOR ISCHEMIA REPERFUSION INJURY:

HK-2 cells were seeded in 6 well culture plates as described previously. Once 80% confluence was reached, growth was arrested by incubation in SFM for 24 hrs. The cells were then incubated with hydrogen peroxide 0.5 mM for 60 minutes at 37°C in an incubator. Cells were then washed with PBS at room temperature three times after the injury period and then incubated in growth media for varying time intervals of 6,12,24,and 36 hours. The control group were not exposed to hydrogen peroxide but were given PBS wash to keep the conditions similar in both groups. The cells were then harvested at the above stated time intervals to assess markers of oxidative stress injury and adaptation as shown in Figure 3.1.

Markers of Oxidative Stress measured include oxidised and total glutathione and protein thiol content of HK-2 cells and Malondialdehyde (MDA) using methods established in our laboratory. Adaptive Enzyme Systems measured include: The activity of the enzymes catalase, glutathione peroxidase, using methods established in our laboratory.

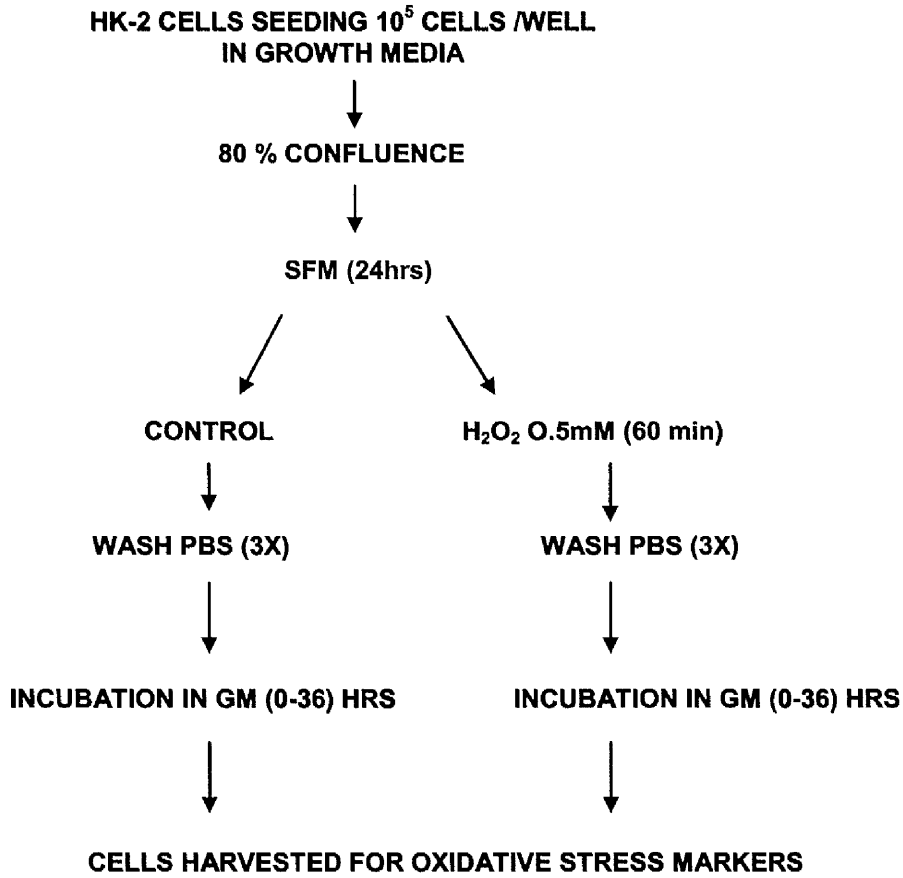


Figure 3.1: Ischemia reperfusion injury model

3.3 MODEL FOR CHEMOKINE INDUCTION:

HK-2 cells were grown as described previously. 80% confluency was achieved and then cells underwent a period of growth arrest for 24 hrs in SFM. Oxidative stress was induced by exogenous hydrogen peroxide and cells were incubated for 60 minutes with 30 and 300 μM hydrogen peroxide. After 60 minutes the cells were washed three times with PBS at room temperature. Cells were incubated in SFM for next 24hrs.The supernatant at this time point was collected as shown in Figure 3.2. The cells were also harvested in NaOH to determine the protein content which was analysed by BCA (Sigma). Supernatant was analysed for IL-8, MCP-1 and RANTES using Elisa Sandwich techniques (R&D Systems).

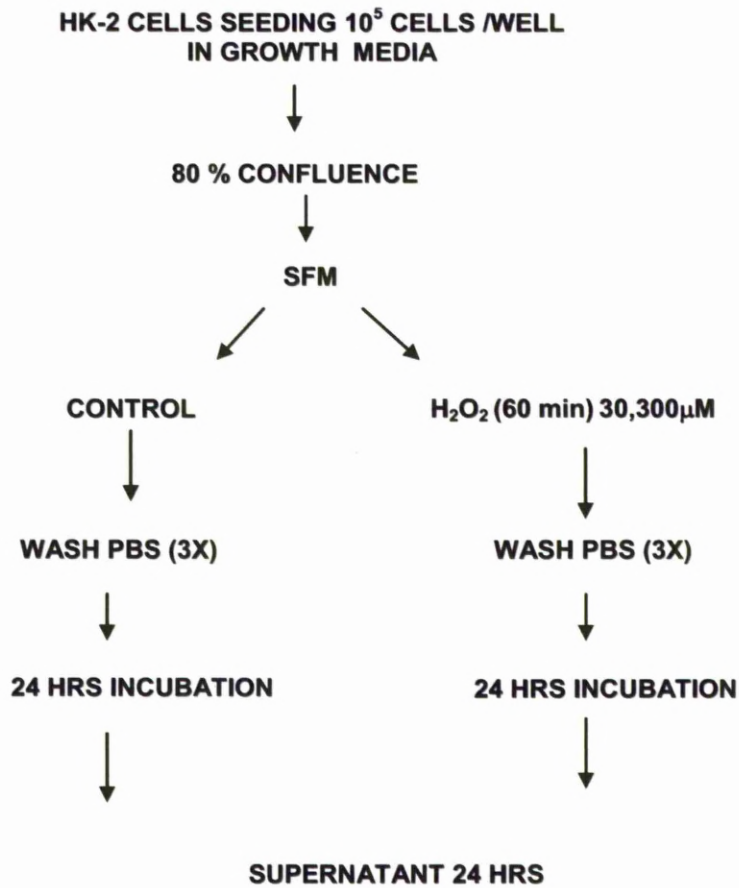


Figure 3.2: Model for chemokine induction

3.4 Model for chemokine suppression with NAC:

HK-2 cells were grown as described previously. 80% confluency was achieved and then cells underwent a period of growth arrest for 24 hrs in SFM. A set of these cells (n=3) were incubated with NAC 10mM for 60 minutes which was washed three times with PBS. The control cells were not exposed to NAC but however received a wash with PBS to keep all conditions similar in both sets. Cells were then incubated in H_2O_2 30 and 300μM for 60 min. After 60 min cells were washed with PBS three times and incubated in SFM for 24 hrs. Supernatant was collected and analysed for IL-8 and MCP-1 using Elisa Sandwich techniques (R&D Systems) as shown in Figure 3.3.

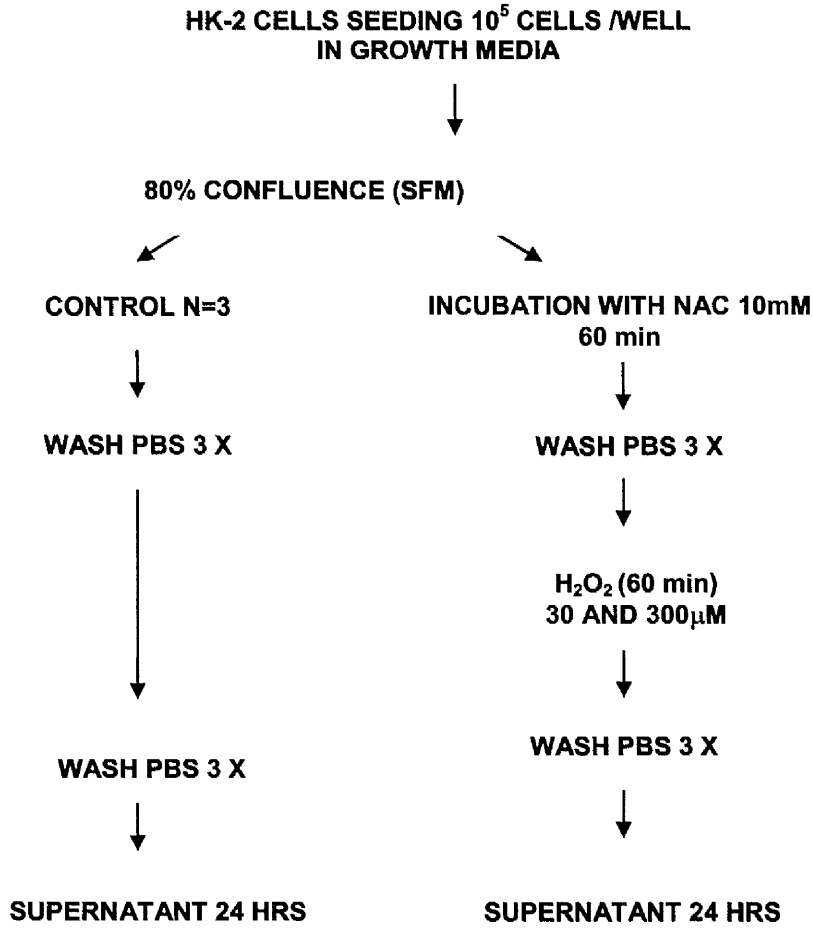


Figure 3.3: Model for chemokine suppression with NAC

3.5 Model for chemokine induction with IL-1 beta and NAC suppression:

HK-2 cells were cultured as described previously. 80% confluency was achieved and then cells underwent a period of growth arrest for 24 hrs in SFM. A set of these cells (n=3) were incubated with NAC 10mM for 60 minutes and washed three times with PBS. The control cells were not exposed to NAC but however received a wash with PBS to keep all conditions similar in both sets. Following this some cells (n=3) were pre-incubated with IL-1β 1ng/ml for 24 hrs.

Other cells remained in SFM. Cells were washed with SFM three times and incubated in H_2O_2 30 and 300 micromoles for 60 min. Again cells were washed with SFM three times and incubated in SFM for 24 hrs. Supernatant was collected and analysed for IL-8 and MCP-1 using Elisa Sandwich techniques (R&D Systems) as shown in Figure 3.4.

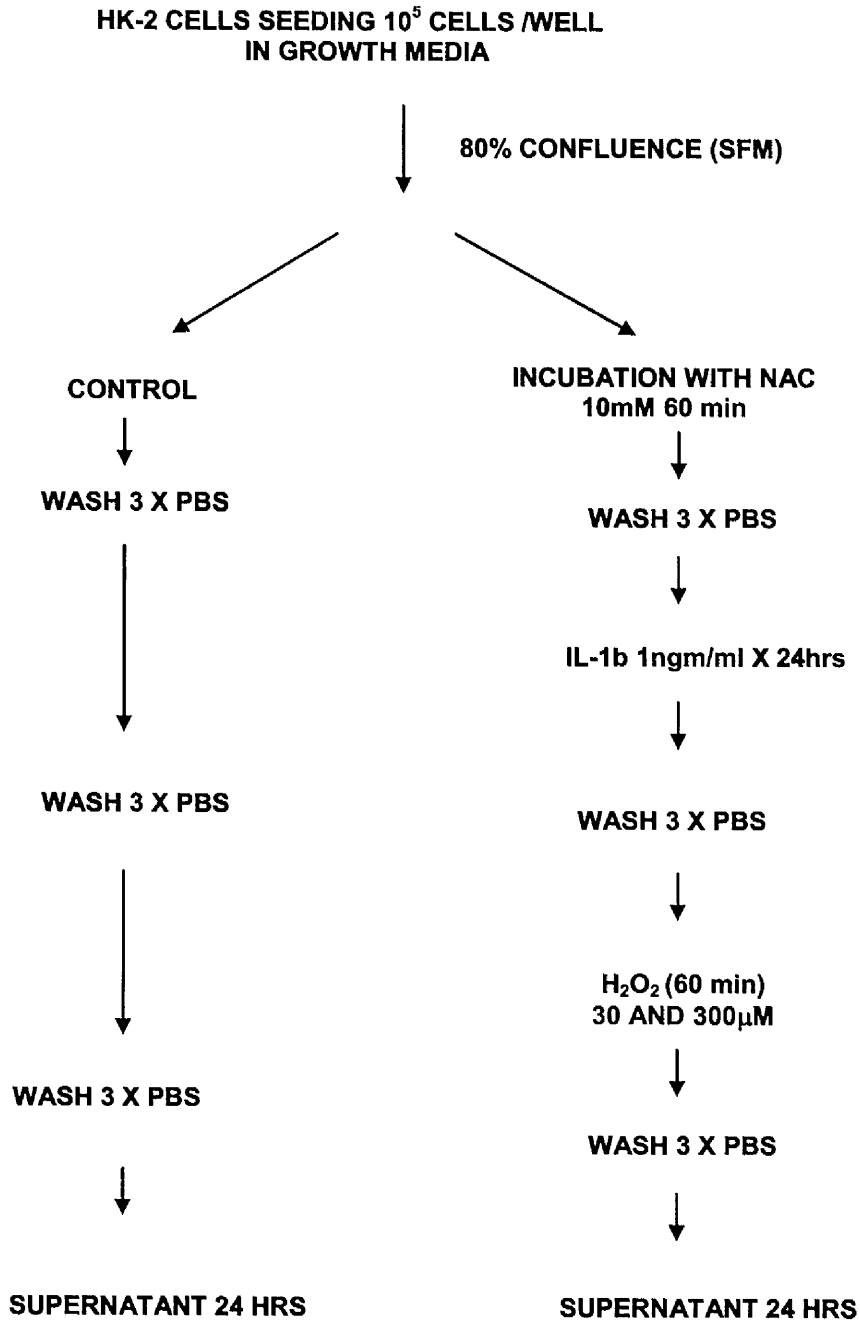


Figure 3.4: Model for chemokine induction with IL-1 beta and NAC suppression:

3.6 ELISA METHODS FOR IL-8 (CXCL8):

The assay for measuring IL-8 released into the cell culture media is based on a quantitative sandwich enzyme immunoassay technique.

3.6.1 Materials

Capture antibody (R&D DY 208)

Detection antibody (R&D DY 208)

Standard (Recombinant IL-8 protein) (R&D DY 208)

Streptavidin-HRP (R&D DY 208)

PBS

Wash buffer (0.05% Tween 20 in PBS)

Block buffer (1% BSA in PBS)

Reagent diluent (0.1% BSA,0.05% Tween 20 in Tris-buffered saline)

Substrate solution (1:1 mixture of Colour reagent A and Colour reagent B) (R&D DY 999)

Stop solution 2N H_2SO_4 (R&D DY 994)

Plate covers

Microplate reader (Powerwave X340, Bio-tech Instruments Inc, Vermont, USA)

3.6.2 ELISA PROTOCOL:

Plate preparation

Capture antibody was diluted in PBS to a working concentration of 4µg/ml. 96 well microplate was coated immediately with 100µl per well. The plate was sealed and incubated overnight at room temperature. Each well was

aspirated and washed with wash buffer three times. The plate was blocked with 300µl of block buffer for 1 hr. Repeat aspirate and wash three times.

3.6.3 Assay procedure:

The standards were prepared by reconstituting the lyophilised powder, provided by the manufacturer (R&D Systems). Serial dilutions were then carried out using the same diluent to provide standards of 1000, 500, 250, 125, 62.5, 31.2 and 15.6pg/ml100ul of sample or standard in reagent diluent was added in each well in duplicate and incubated for 2hrs. The process of repeat aspirate and wash with wash buffer was performed three times. 100 µl of detection antibody was added and incubated for 2 hrs. The process of repeat aspirate and wash as above was done three times again. 100 µl of Streptavidin-HRP was added for 20 minutes in dark and process of aspirate and wash repeated as above. 100 µl of substrate solution was added and incubated for 20 minutes in dark to which 50µl of stop solution was added.

3.6.4 Calculation of results:

The plate was read using a microplate reader (Powerwave X340, Bio-tech Instruments Inc, Vermont,USA) at 450nm using background subtraction at 570nm. The concentrations of the unknown samples were determined from the standard curve obtained and multiplied by the dilution factor to obtain the concentration. These results divided by the total protein concentration (mg/ml) gave the total IL-8 concentration in pg/mg protein.

3.7 ELISA METHODS FOR MCP-1 (CCL2):

3.7.1 Materials:

MCP-1 Microplate – 96 well polystyrene microplate coated with mouse monoclonal antibody against MCP-1

MCP-1 Conjugate

Standard (Recombinant human MCP-1)

Calibrator diluent (RD5L) Concentrate 5X

Wash buffer concentrate

Colour reagent A

Colour reagent B

Stop solution 2N H_2SO_4

12mmx75mm polypropylene test tubes

Plate covers

Microplate reader (Powerwave X340, Bio-tech Instruments Inc, Vermont, USA)

3.7.2 ELISA PROTOCOL:

Reagent preparation

All reagents brought to room temperature before use 20ml Wash buffer concentrate diluted with deionised water to prepare 500ml of wash buffer 20ml of Calibrator diluent (RD5L) diluted with deionised water to yield 100ml MCP-1 standard is reconstituted with 5ml of calibrator diluent yielding a stock solution of 2000pg/ml. Allow standards to sit for 15 minutes before making a serial dilution as shown in Figure 3.5.

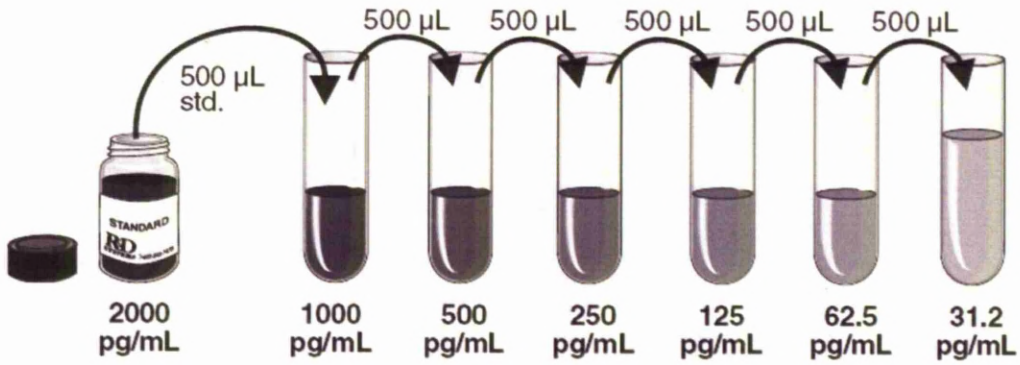


Figure 3.5: Showing serial dilution of MCP-1 (CCL2) standard

3.7.3 Assay procedure

200ul of standard and sample was added in each well and incubated for 2 hrs. Aspiration and wash of each well was done using wash buffer at least three times. 200ul of MCP-1 Conjugate was then added to each well and incubated for 1hr. The process of aspiration and wash was repeated three times again. 200µl of substrate solution was then added to each well and incubated for 20 minutes in dark. 50µl of stop solution was then added to each well.

3.7.4 Calculation of results

The plate was read using a microplate reader (Powerwave X340, Bio-tech Instruments Inc, Vermont, USA) at 450nm using background subtraction at 570nm. The concentrations of the unknown samples were determined from the standard curve obtained and multiplied by the dilution factor to obtain the concentration. These results divided by the total protein concentration (mg/ml) gave the total MCP-1 concentration in ng/mg protein.

3.8 Viability:

Cell viability was assessed using the Trypan blue exclusion assay. Adherent and floating cells were harvested at 24 and 48 hrs after exposure to either SFM alone (control) or to Hydrogen peroxide preparations at concentration of 30,300 and 1000 μM. The number of live and dead cells (stained blue) was counted, and the viability was expressed as the percentage of live cells within the total number of cells counted.

3.9 CLINICAL STUDIES

Transplant graft biopsies were collected from patients who were undergoing the biopsy procedure due to graft dysfunction, the common indications being DGF, Acute cellular rejection, drug toxicity and CAN. An informed written consent was procured from all patients. The procedure was done under local anaesthesia according to the unit protocol under ultrasound guidance. An automatic biopsy gun was used with 16Fr true cut needle. One core of tissue was taken for research purposes only if it was safe enough to proceed for an extra core and the patient was comfortable and once again gave a verbal consent. Some biopsy samples were procured from deceased donor kidneys prior to implantation only when the family had given consent for the organ to be used for research purposes. Biopsy specimen was snap frozen immediately in liquid nitrogen and stored at -70°C until further analysis. Samples were later processed for western blot and tested for markers of oxidative stress.

3.9.1 Renal transplant biopsy data:

3.9.2 Western blot:

Biopsy specimens were taken out from the -70°C freezer and ground mechanically in liquid nitrogen. Homogenisation buffer (1% SDS, PI 0.5ML, and EGTA 9.5mg) was added and the mixture was centrifuged at 16000 g at 4°C for 10 minutes; the resulting supernatants were stored at –70°C. The protein concentration of the sample was determined using the BCA method. Aliquots of samples containing 40 µg of total protein were heated for 5 min at 95°C in denaturing loading buffer. The proteins were then resolved on 4% and 12.5% SDS-PAGE mini gels and blotted onto nitrocellulose membranes. The membranes were blocked for 1 hr at room temperature in Blotto (5% skimmed milk in PBS) supplemented with 0.05% Tween 20 (PBST) and exposed at room temperature to primary antibodies using antibody dilutions and time period as recommended by the manufacturer. The membranes were repeatedly washed in PBST and exposed at room temperature to secondary antibodies as recommended by the manufacturer. Protein bands were visualized using Super Signal West Dura Extended duration chemiluminescent substrate (Pierce/Perbio Science) and recorded using a Chemidoc XRS imaging system and Quantity one software (Bio-Rad). The intensity of the signal from each band was corrected for background signal and expressed as a percentage of the content of control cells.

3.9.2.1 Reagents

Laemmli buffer: 46.03 mg/ml SDS, 20.9% glycerol, 2.1% (v/v) β-mercaptoethanol, 0.052 mg/ml bromophenol blue in 0.128M Tris/HCl buffer, pH 6.8.

3.9.2.2 Protocol

One hundred micrograms of total protein was added to an equal volume of Laemmli buffer. Samples were boiled for 5min and then allowed to cool to room temperature. Samples were loaded onto a polyacrylamide gradient gel alongside a molecular weight rainbow marker (Amersham Biosciences, UK).

3.9.2.3 Preparation of polyacrylamide gradient gels

Reagents

- Stock acrylamide solution: 30% acrylamide, 0.8% bisacrylamide cross-link, in dH_2O (Protogel, National Diagnostics, USA)
- Gel buffer: 1.5M Tris/HCl, 0.384% SDS, pH 8.8. (Protogel buffer, National Diagnostics, USA)
- Stacking buffer: 0.5M Tris/HCl, 0.4% SDS, pH 6.8. (Protogel stacking buffer, National Diagnostics, USA)
- 12% acrylamide solution (100ml): 40ml stock acrylamide solution, 26ml gel buffer, 32.9ml dH_2O
- 4% stacking gel solution (100ml): 13ml stock acrylamide solution, 25ml stacking buffer, 61ml dH_2O
- 10% (w/v) Ammonium persulphate (APS)
- NNN'N'-tetramethylethylene-diamine (TEMED)

Protocol

12% acrylamide was prepared as described above. One hundred microlitres of 10% APS and 10µl of TEMED was added to 10ml of 12% acrylamide solution to catalyse gel formation. The gel solution was poured between glass plates (8x10cm) with 2mm spacers and allowed to set for 10-15mins.

The 4% stacking acrylamide solution was prepared as described above. One hundred microlitres of 10% APS and 25µl of TEMED was added to 10ml of 4% acrylamide solution. The 4% gel solution was poured on top of the 12% gel to form a 1-1.5cm stacking gel and a well comb was placed in position to facilitate sample loading.

3.9.2.4 Electrophoresis of proteins

Reagents

- Electrophoresis buffer: 10X Tris/Glycine/SDS (0.025M Tris, 0.192M glycine, 0.1% (w/v) SDS; National Diagnostics, USA)

Protocol

Electrophoresis was carried out using an Anachem Electrophoresis tank with 1X electrophoresis buffer. Electrophoresis was carried out at a constant current of 20mA per gel until samples had run through the 4% stacking gel; the current was then increased to 40mA per gel until the 15KDa molecular weight marker had reached the bottom of the gel. The tank was cooled with H_2O during electrophoresis.

3.9.2.5 Western blotting of separated proteins

Reagents

- Anode 1 buffer: 0.3M Tris in a 20% methanol solution, pH 10.4.
- Anode 2 buffer: 25mM Tris in a 20% methanol solution, pH 10.4.
- Cathode buffer: 40mM 6-amino n hexanoic acid in a 20% methanol solution, pH 7.6.

Protocol

The Multiphor II discontinuous blotting system (Pharmacia, Milton Keynes, UK) was used to transfer proteins to a nitrocellulose membrane. This system consists of two graphite plate electrodes. Following electrophoresis gels were removed from the glass plates and the 4% stacking gel was removed. The gel was placed on top of a nitrocellulose membrane and this was sandwiched between the electrodes with Whatman No 1 filter paper pre-soaked in anode and cathode buffers as shown in Figure 3.6. A constant current of 0.8mA/cm^2 was applied to the system for 1.5hrs.

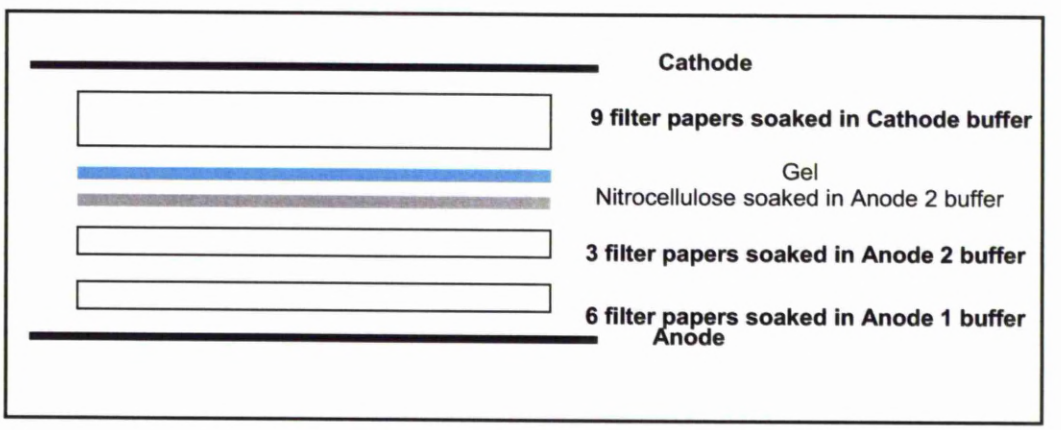


Figure 3.6: Schematic diagram of western blotting

3.9.2.6 Probing of nitrocellulose membrane for protein content

Reagents

- PBS solution: 0.05M KH_2PO_4, 0.05M Na_2HPO_4, 1.3M NaCl in dH_2O, pH 7.2.
- PBS solution: 0.05M KH_2PO_4, 0.05M Na_2HPO_4, 1.3M NaCl in dH_2O, pH 6.0.

- PBS/Tween solution: 0.05% (v/v) polyoxyethylene-sorbitan monolaurate (Tween 20) in PBS solution.
- Blocking solution: 5% (w/v) powdered milk in PBST

Protocol

Following transfer, the nitrocellulose membrane was removed and placed in 50ml of a blocking solution for either 1 hour at room temperature or overnight at 4°C. Membranes were then washed in PBST solution. The nitrocellulose membrane was then analysed for oxidative stress markers using a range of monoclonal or polyclonal antibodies. The nitrocellulose was agitated for an hour in 10ml of a solution of PBST containing the primary antibody at the concentration listed in Table 3.1. The membrane was then washed in PBST solution for 3x10mins and placed into the appropriate peroxidase labelled secondary antibody solution in PBST containing 25% FCS as a non-specific blocking agent 1 hour at room temperature. The membrane was then washed for 3x10mins in PBST and then for a further 15min in PBS pH6.0 as this is the optimal pH for the peroxidase activity. The membrane was developed using Super Signal West Dura chemiluminescent detection kit (Pierce, Rockford, USA). The membrane was placed between acetate sheets and analysed using a Bio-Rad Chemi-XRS System and Quantity One software (Bio-Rad, Hercules, USA).

Table 3.1: The primary and secondary antibodies used in Western blotting

Antibody	Source	Species	Primary antibody dilution	Incubation Time/Temp	Secondary antibody dilution
Trx (2B1) Thioredoxin SC-58439	Santa Cruz biotechnology	Mouse monoclonal	1:100	1.5 hrs/RT	Anti-Mouse 1:5000
TrxR1 (A-20) Thioredoxin reductase SC-18220	Santa Cruz Biotechnology	Goat polyclonal	1:200	2hrs /RT	Anti goat 1:2000
Anti- Cu/Zn SOD SOD-100	Stressgen	Rabbit polyclonal	1:4000	2hrs /RT	Anti Rabbit 1:5000
Glutathione peroxidase Ab8850	Abcam	Sheep polyclonal	1;1000	2hrs /RT	1:1000 Anti sheep
Thioredoxin T8690	Sigma	Human T- cell, recombinant			
Thioredoxin2 Ab16836	Abcam	Rabbit polyclonal	1:1000	2hrs/ RT	Anti Rabbit 1;5000
Anti-catalase C 0979	Sigma	Mouse Monoclonal	1:2000	2hrs/ RT	Anti mouse 1:10000
Anti- MnSOD SOD 110	Stressgen	Rabbit polyclonal	1;4000	2hrs/ RT	Anti rabbit 1:5000
Beta actin Ab8224	Abcam	Mouse monoclonal	1: 5000	1hr/ RT	Anti mouse 1:5000

3.9.2.7 Primary Antibodies

Trx (2B1)

Thioredoxin is a mouse monoclonal antibody (Santa Cruz biotechnology, USA) catalogue number SC-58439. This antibody is raised against the full length of thioredoxin of human origin; molecular weight 12 kDa.

TrxR1 (A-20)

Thioredoxin reductase is a goat polyclonal (Santa Cruz Biotechnology, USA) catalogue number SC-18220. It is recommended for detection of precursor and mature thioredoxin reductase of human and to lesser extent rat origin; molecular weight 55 kDa.

T8690

Human T- cell recombinant Thioredoxin (Sigma-Aldrich, Dorset, U.K) catalogue number T 8690. It is cloned from Jurkat cell cDNA and has a molecular weight of 14kDa.

ab16836

Thioredoxin2 is a rabbit polyclonal antibody (Abcam) catalogue number ab16836. It reacts against human and mouse species. It is localized in the mitochondrion and has a molecular weight of 18 kDa.

SOD 100

Anti- Cu/Zn SOD is a purified rabbit polyclonal antibody that detects Cu/Zn superoxide dismutase (Stressgen Inc, Canada). It is recommended for use in human samples and has a molecular weight of 23 kDa.

SOD 110

Anti-MnSOD is a rabbit polyclonal antibody (Stressgen Inc, Canada) that detects MnSOD; molecular weight 25 kDa.

ab8850

Glutathione peroxidase is a sheep polyclonal antibody (Abcam) purified from human erythrocytes and reacts with human glutathione peroxidase with bands seen at 23 and possibly 92 kDa.

C 0979

Anti-catalase is a mouse monoclonal antibody derived from CAT 505 hybridoma (Sigma-Aldrich, Dorset, U.K). It reacts specifically against human catalase; molecular weight of 60kDa.

3.9.2.8 Removing antibodies and re-probing the nitrocellulose membrane

Reagents

- 100mM β-mercaptoethanol (Sigma-Aldrich, Dorset, U.K.)
- 2% (w/v) SDS
- 62.5mM Tris/HCl, pH 6.7
- PBS/Tween solution, pH 7.2
- Blocking solution containing 5% (w/v) powdered milk PBST

Protocol

Antibodies were removed from the nitrocellulose membrane and the membrane was re-analysed for other proteins. After exposure to chemiluminescent detection solution (Pierce, Rockford, USA), the membrane was washed in PBST solution for 5mins and then incubated in a solution containing 100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris/HCl, pH 6.7 for 30mins at 50°C. The membrane was then washed for 2x10mins in PBST solution at room temperature. The membrane was then placed in 50ml of blocking solution for 1hr at room temperature or alternatively at 4°Covernight. The membrane was then analysed for the content of other oxidative stress markers as described above in section 4.2.6.

3.9.3 Ethical approval:

Ethical approval was granted by the Liverpool (Adult) local research ethics committee. REC reference number 01/189, study number 2226. Permission for use of clinical biopsies was taken from the patients undergoing renal transplant biopsies for any clinical indication after signing an informed consent form.

3.9.4 Statistical analysis

Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS version 11.01, Surrey, UK). All data were expressed as means ± SE. Data were analyzed using a one-way repeated-measures ANOVA. Where a significant value was observed, Tukey's HSD post hoc analysis was performed to identify where the significant differences occurred. A P value of <0.05 was considered significant.

CHAPTER 3

RESULTS: LABORATORY MODEL

4.1 Oxidative stress model

4.1.1 Glutathione peroxidase activity

HK-2 cells were cultured in six well plates and upon 80% confluence they were growth arrested. Oxidative stress was introduced by incubation with 0.5mM of hydrogen peroxide for 1 hour. The activity of antioxidant enzyme glutathione peroxidase was measured spectrophotometrically. Measurements were made at the time intervals of 2,4,8,12,24 and 36 hours. The results were expressed in mU per mg of protein. There was a significant rise in glutathione peroxidise activity in the samples collected after 36 hours of injury ($p<0.05$) as shown in Figure 4.1. The median activity in control samples was 95.34 mU/mg (SE23.88) while it was 181.8 mU/mg (SE10.61) in test samples at 36 hours. All experiments were repeated on at least three separate occasions. The results show that hydrogen peroxide was able to induce direct oxidative stress, to which the HK-2 cells responded by activation of antioxidant enzyme Glutathione peroxidise.

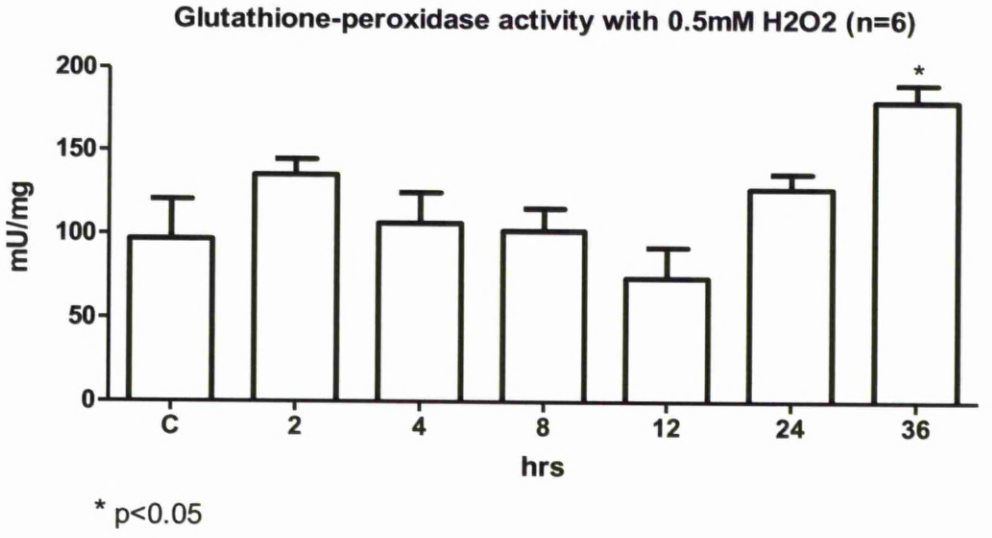


Figure 4.1: Glutathione peroxidase induction with hydrogen peroxide (n=6)

4.1.2 Catalase activity

Similar to the above experiment, HK-2 cells were exposed to 0.5mM hydrogen peroxide for one hour and cells were harvested at time intervals of 2,4,8,12 and 36 hours to detect the antioxidant enzyme catalase spectrophotometrically as described earlier. There was no significant change in the activity of catalase at all time intervals.(n=12) as shown in Figure 4.2. There could be several reasons for the lack of induction of catalase enzyme. In our laboratory catalase was often seen to respond poorly to stimulation in HK-2 cells. Our initial experiments were performed in porcine LLCK cell line where catalase had shown favourable response to stimulation. Other reasons maybe due to inactivation of catalase by hydroxyl radicals while GPx maybe less affected (Pigeolet et al 1990). The concentration of hydrogen peroxide used may also affect the oxidative damage. The micromolar concentration maybe more effective while we had used milimolar concentration in this experiment.

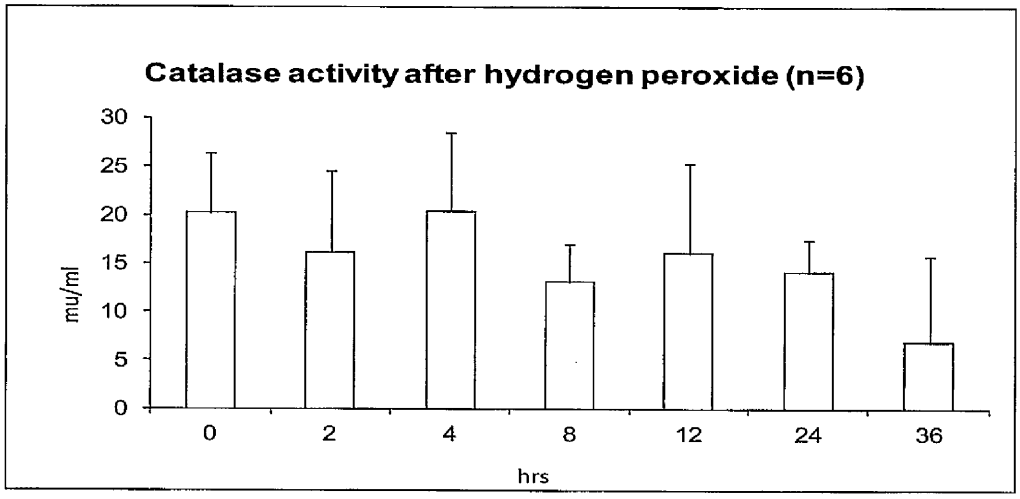


Figure 4.2 Catalase activity seen after hydrogen peroxide (n=6)

4.2 Chemokine model

4.2.1 IL-8 (CXCL8) experiments

4.2.1.1 Basal secretion After sub-culturing the HK-2 cells in 6 well plate once 70-80% confluence was reached cells were growth arrested by incubating in serum free media. The supernatant was then collected at intervals of 6,12, 24 and 48 hrs. IL-8 (CXCL8) was detected within 6 hours of incubation 70.3pg/ml (SE36.86) and the concentration increased exponentially with time to 456.8pg/ml (SE 94.28) at 12 hours, 925pg/ml (SE139.1) at 24 hours and 1337pg/ml (SE 158) at 48 hours as shown in Figure 4.2. Il-8 production from cells that were not stimulated could be due to the stress of cell culture, transfer of cells and change in media. The exponential rise with time is possibly the effect of accumulation.

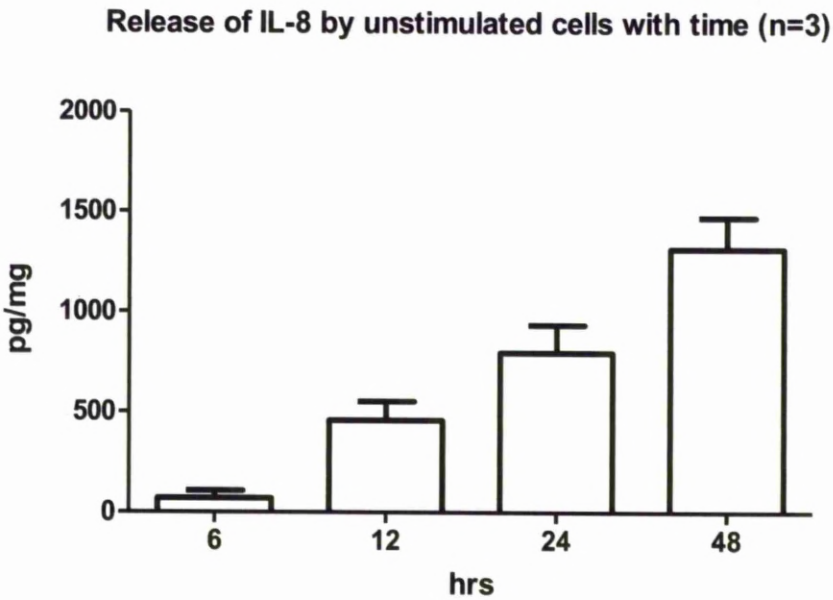
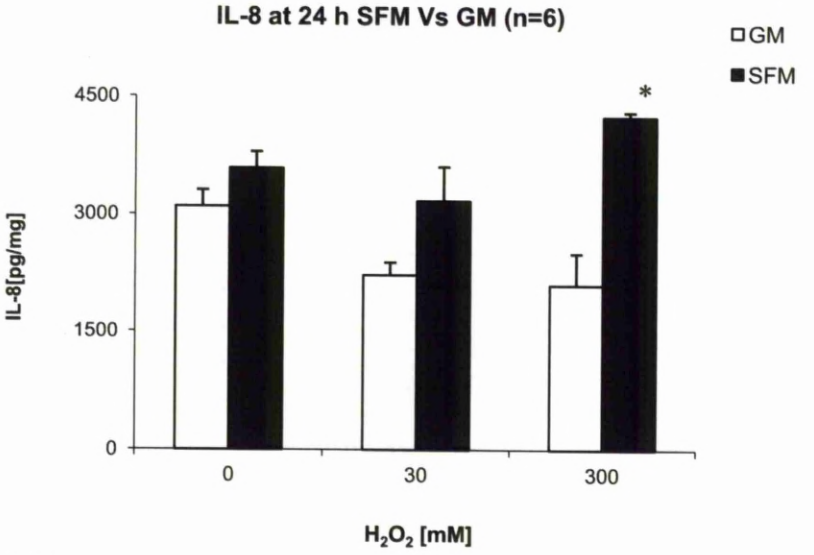


Figure 4.3: HK2 cells showing basal secretion of IL- 8 (CXC) with time

4.2.1.2 Effect of Hydrogen peroxide on IL-8 (CXCL8) production

The HK-2 cells were incubated with 30 and 300 μM of hydrogen peroxide in growth media and serum free media separately. No change in IL-8 (CXC) was seen in cells which were cultured in growth media (enriched with fetal calf serum) that were induced with 30 or 300 μM of hydrogen peroxide. In comparison, cells incubated in serum free media induced with 300μM of hydrogen peroxide showed a significantly increased production of IL-8 (CXCL8) which was measured at 24 hrs, ($p<0.05$) as shown in Figure 4.4.

The cells could be less stressed in growth media as compared to serum free media thus resulting in less response in growth media group. Also the albumin present in fetal calf serum may have scavenged the free radicals resulting in poor response in growth media group.



*P<0.05

Figure 4.4: IL-8 induction with 300μM H_2O_2 in SFM but not in GM at 24 hrs

4.2.1.3 Effect of Interleukin-1beta on IL-8 (CXCL8) production

HK-2 cells were incubated with Interleukin-1beta (1ng/ml) for 24 hours and supernatant collected after 24 hours showed increased production of IL-8 (CXCL8) to a significant value of 7989pg/ml(SE534.9) ($p<0.05$). In the same experiment 30 and 300 μM of hydrogen peroxide also caused increased production of IL-8 to significant levels ($p<0.05$). In a separate sub-group of cells which were incubated with Interleukin-1beta and then induced with 30 and 300μM hydrogen peroxide a further increase in the production of IL-8 (CXCL8) was seen as shown in Figure 4.5 ($p<0.05$). There was no statistical difference between the rise with 30 and 300 mM hydrogen peroxide alone but the rise between Interleukin-1beta stimulated cells followed by incubation with 30 and 300 mM hydrogen peroxide group was significant suggesting synergism between Interleukin-1beta and hydrogen peroxide. These results were consistent in supernatant collected both at 6 and 24 hrs. (Figure 4.6) Cytokines like IL-1β are known to increase IL-8 (CXCL8) production. Hydrogen peroxide is also known to increase IL-8 release and both may act through separate signal pathways affecting the IL-8 production. When both signal pathways are involved it can lead to synergistic increase in IL-8(CXCL8) production.

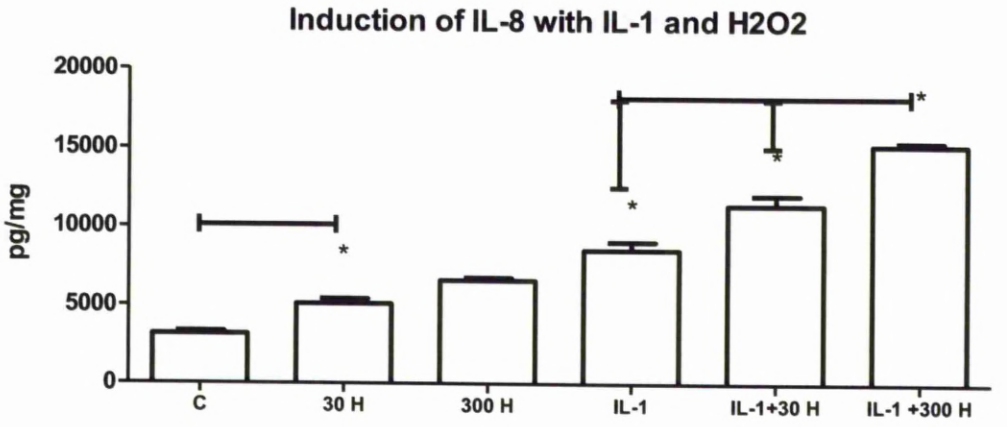


Figure 4.5: IL-8 induction with IL-1 alone and in combination with H_2O_2(n=6)
* = P <0.05

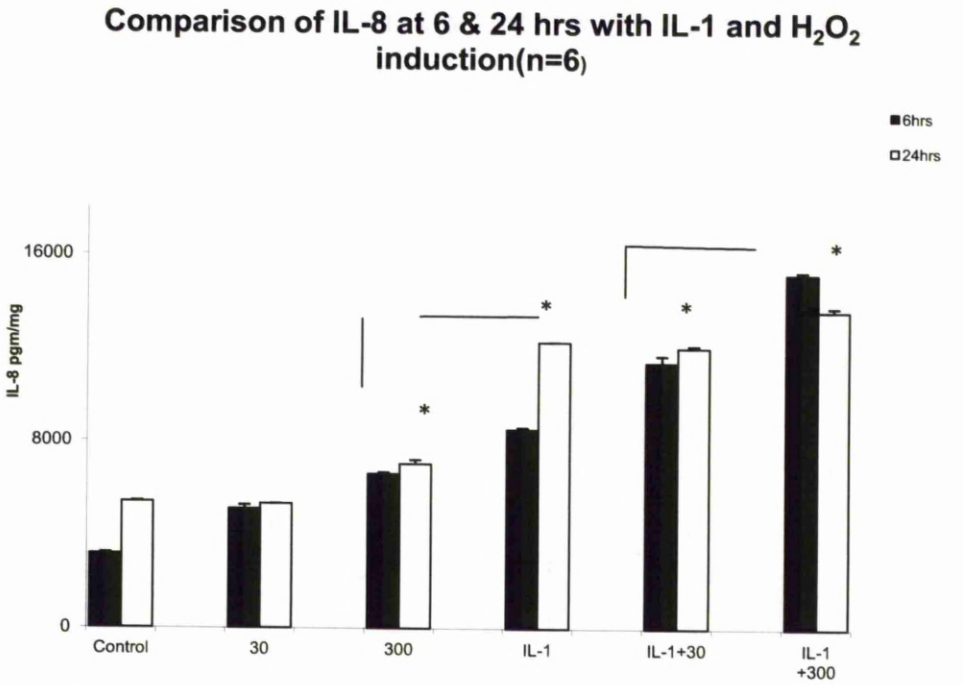


Figure 4.6: IL-8 induction with IL-1 alone & combined with H_2O_2 at 6 & 24hrs
*= p<0.05

4.2.1.4 Effect of N-Acetyl cysteine on Hydrogen peroxide induction:

HK-2 cells were pre-incubated with NAC 10mM for 60 minutes. Following this the cells were stimulated with hydrogen peroxide 30 and 300 μM. IL-8 (CXCL8) was inhibited in both 30 and 300 mM groups 4464pg/ml (SE425.5) and 3993pg/ml (SE1464)

respectively as compared to hydrogen peroxide alone groups 5919pg/ml (SE658.7) and 6865pg/ml (SE105.5) as shown in Figure 4.7 however this did not reach any statistical significance. An interesting paradoxical observation was seen in the NAC alone group as IL-8(CXCL8) levels were slightly elevated in this group. NAC in high dose can result in oxidative stress itself.

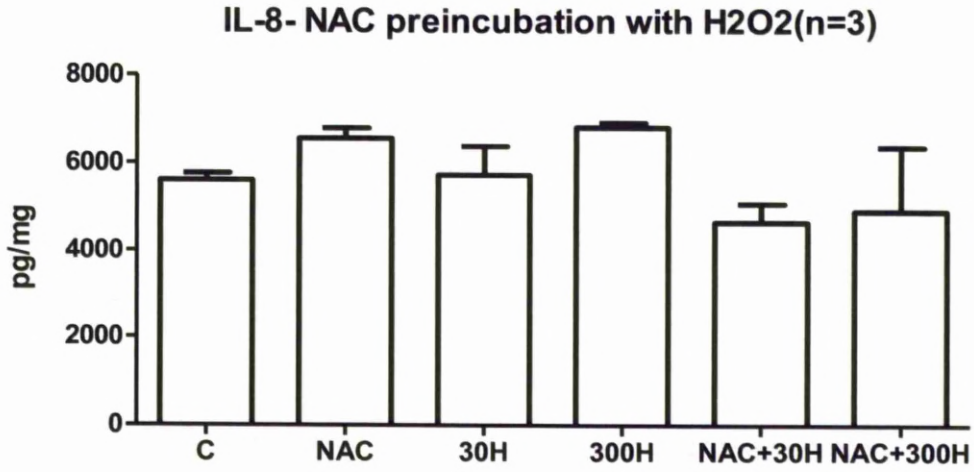


Figure 4.7: NAC pre-incubation blocking the effect of H_2O_2 on IL-8 (CXCL8)

There were no statistical differences between data points (P>0.05)

4.2.1.5 Interleukin-1beta alone and with N-acetyl cysteine:

HK-2 cells when stimulated with Interleukin-1beta 1ng/ml caused significantly increase in the production of IL-8 7990pg/ml (SE 972.2) when compared with cells that were incubated with culture medium alone 5631pg/ml (SE 154.3)($p<0.05$). Further, if the cells were pre-incubated with NAC 10mM the induction of IL-1 β could be significantly suppressed 5669pg/ml (SE 797.6) $p<0.05$ as shown in Figure 4.8. This experiment proves that IL-1β mediated IL-8 production is partly mediated via oxidative radicals as NAC a powerful antioxidant was able to ameliorate this reaction.

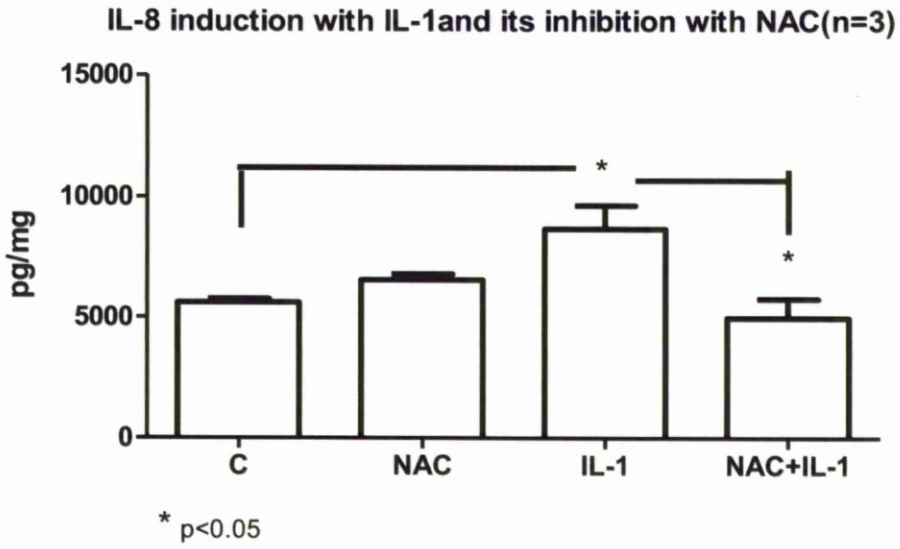


Figure 4.8: Interleukin-1beta induction of IL-8 (CXC) and its inhibition by pre incubation with NAC

4.2.1.6 NAC inhibition of IL-8 (CXCL8) in cells stimulated with both H_2O_2 and IL-1β

The above experiments were repeated on a sub-group of cells that were induced with IL-1β and also hydrogen peroxide 30 and 300 μM. There was an expected and significant increase in IL-8 with IL-1β 8341pg/ml (SE 711.1) and NAC was able to suppress this partially 5500pg/ml (SE 291.5). NAC was also able to inhibit IL-8 (CXCL8) in cells that were incubated with IL-1 β and hydrogen peroxide (30 and 300μM). The subgroup that was stimulated with 30μM of hydrogen peroxide were seen to inhibited with 10mM NAC but not significantly 2495pg/ml (SE 923.7) vs 3222pg/ml (SE904.3). The subgroup that was stimulated with 300μM of hydrogen peroxide were inhibited significantly by 10mM NAC, 1961pg/ml SE(321.7) vs 4837pg/ml (SE414.4) as shown in figure 4.9. This result proves the role of oxidative stress in mediating IL-8(CXCL8) release with hydrogen peroxide and shows the dose related response.

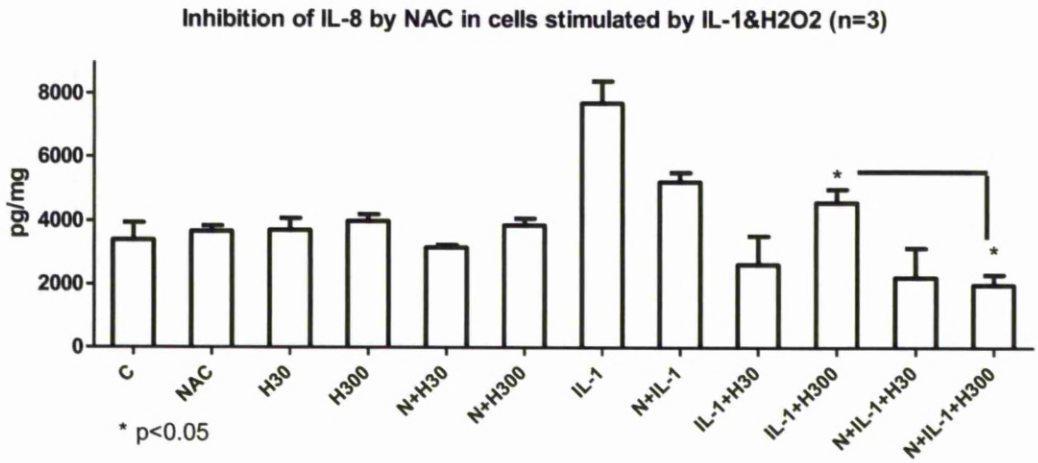


Figure 4.9: Combined effect of NAC pre-incubation on IL-1 and H_2O_2 stimulated cells, N = NAC

4.3 MCP-1 (CCL2):

4.3.1: Induction of MCP-1 (CCL2) with Hydrogen peroxide and Interleukin-1beta

Similar to experiments on IL-8 (CXCL8), HK-2 cells were incubated with 30μM and 300 μM of hydrogen peroxide for 60 minutes in SFM. Supernatant was collected at 24 hrs. There was no significant change in MCP-1 (CCL2) concentration with hydrogen peroxide. In the same experiment when cells were incubated with IL-1β they showed a significant increase in MCP-1 (CCL2) 2902pg/ml (SE 67.60) compared to the control group, 1616pg/ml (SE 54.54) as shown in Figure 4.10. These results indicate that hydrogen peroxide has less involvement in MCP-1(CCL2) induction as compared to IL-8 (CXCL8), however Interleukin -1β was able to potentiate its release suggesting different mechanisms maybe involved.

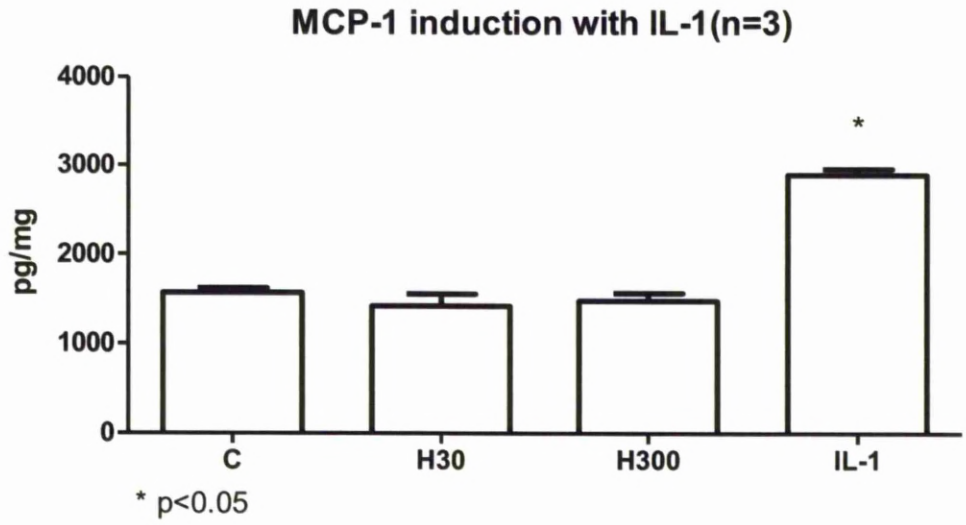


Figure 4.10: Induction of MCP-1 (CCL2) with hydrogen peroxide and IL-1β

4.3.1.2 MCP-1 (CCL2) induction with Hydrogen peroxide and IL-1β and inhibition by NAC:

In a separate experiment HK-2 cells could be induced with IL-1β to produce MCP-1, 2902pg/ml (SE 67.60) ($p<0.05$) but not with hydrogen peroxide (30 and 300 μM) as seen in the previous experiment. Pre-incubation with 10mM NAC resulted in inhibition of MCP-1 (CCL2) in the group induced by 300 μM of hydrogen peroxide to 793.7pg/ml (SE 56.60) but not in 30 μM hydrogen peroxide group, 1172pg/ml (SE 109.2) ($p<0.05$). This suggests oxidative stress involvement which is possibly more complex involving several other pathways. Pre-incubation of cells with NAC also resulted in inhibition of IL-1β induction although this was not statistically significant as shown in Figure 4.11.

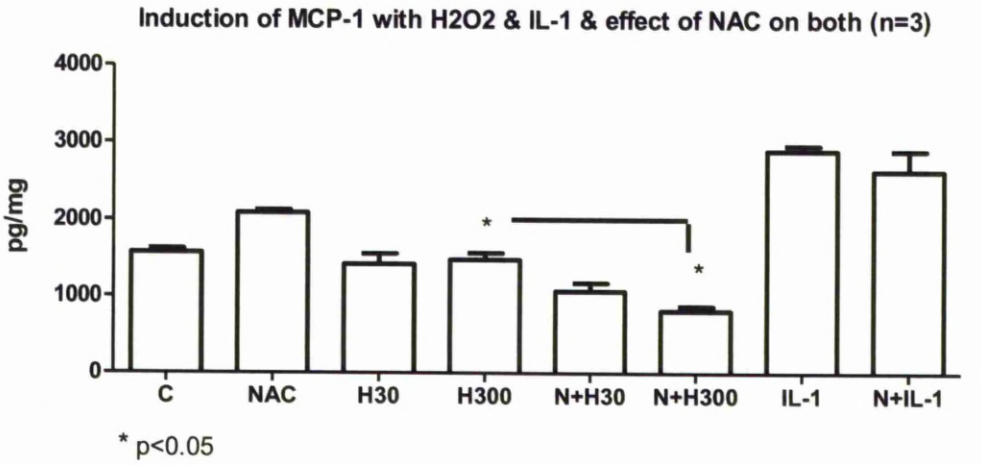


Figure 4.11: MCP-1 (CCL2) induction with Hydrogen peroxide and IL-1β and effect of NAC

4.3.1.3 Combined effect of H_2O_2, IL-1β, and NAC on MCP-1 (CCL2):

The above experiments were repeated similar to the experiment with IL-8 (CXCL8) with the addition of a group which was stimulated with IL-1β and hydrogen peroxide (30 and 300 μM) and a subset was pre-incubated with 10mM NAC. In repeat experiments results were similar to before. IL-1β significantly increased the MCP-1 (CCL2) release to 2902pg/ml (SE 67.60) and NAC was able to inhibit to 2818pg/ml (SE 260.8) although again this increase was not statistically significant. In the subset which were induced with hydrogen peroxide and IL-1, NAC pre-incubation produced a reduction of MCP-1 (CCL2) in 300 μM group to 1044pg/ml (SE 303.4) when compared with the set which did not get NAC, 2636pg/ml (SE 177.4) ($p<0.05$). The group which were incubated with 30μM did not show any significant change as shown in Figure 4.12. Again these results show the involvement of oxidative stress in induction and suppression of MCP-1(CCL2).

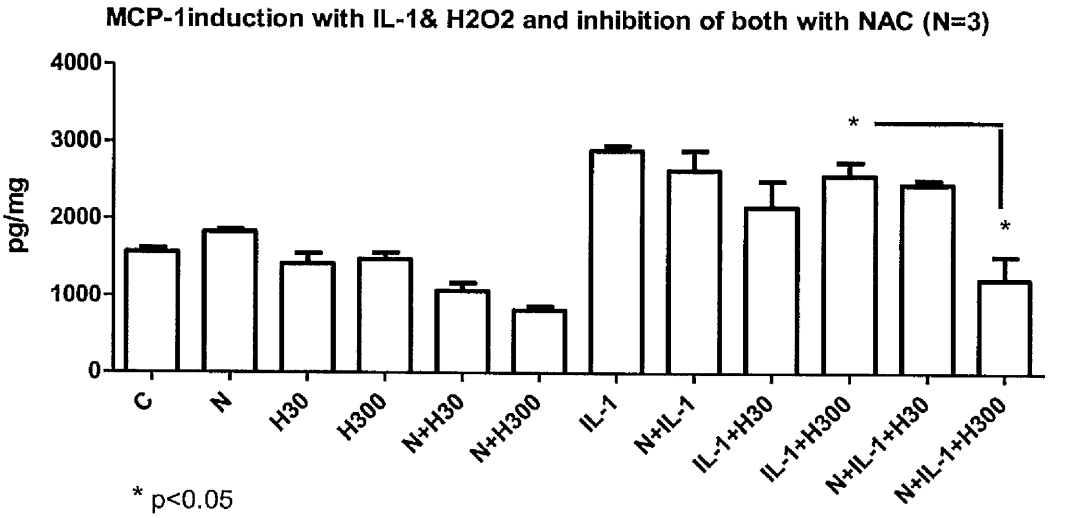


Figure 4.12: Combined effect of NAC pre-incubation on IL-1β and H_2O_2 stimulated cells

4.4 Cell Viability:

Cell viability was assessed using the Trypan blue exclusion assay. Adherent and floating cells were harvested at 24 and 48 hrs after exposure to either SFM alone (control) or to Hydrogen peroxide preparation. The pooled adherent and nonadherent cells remained viable both at 24 and 48 hours after exposure to 30,300,1000 μM hydrogen peroxide with no significant difference between the three groups as shown in figure 4.13.

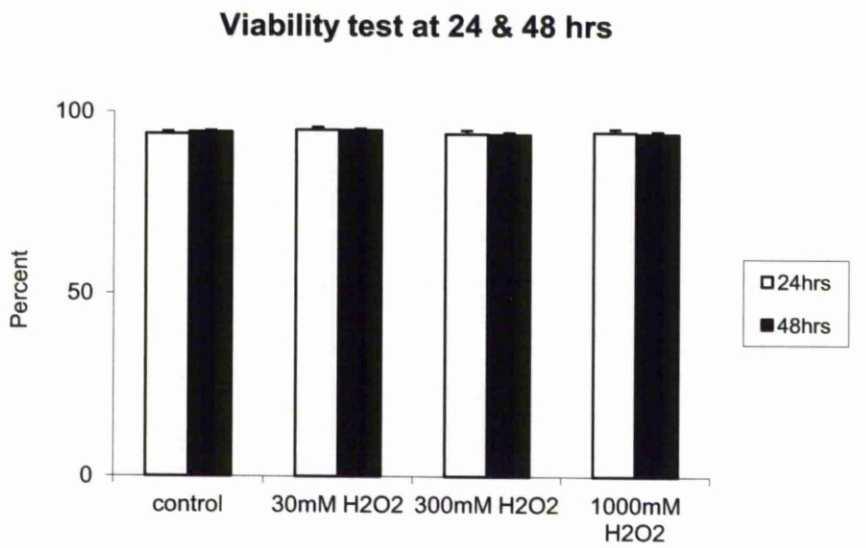


Figure 4.13: Viability test at 24 and 48 hrs with hydrogen peroxide

CHAPTER 4

RESULTS: CLINICAL MODEL

61 renal allograft biopsies were collected from renal transplant recipients who were undergoing this procedure for various clinical reasons. 47 of these biopsies were analysed and their data is presented. Biopsy samples that were not included in the analysis were used in initial trial runs and some were lost during processing. 21 pre-implantation biopsies were procured from deceased donor kidneys. 17 of these were included in the final analysis as the rest were used in initial trial runs. Data is shown in Table 5.1.

Table 5.1: Showing the number of donor and allograft biopsies

	Donor biopsies	Allograft biopsies	Total
Number of biopsies collected	21	61	82
Number of biopsies analysed	17	47	64

5.1 Demography of renal transplant recipients

There were 20 (42.6%) females and 27 (57.4%) males. Median age of the recipients was 44.6 yrs ± 2.13 (19.4-76.9). 35 (74.5%) patients underwent renal transplantation from a deceased donor while the remaining 12 (25.5%) underwent a living donor renal transplantation.11 patients had a first renal transplant from a living donor. 1 patient had a third renal transplant from a living donor. 29 patients had their first renal transplant from a deceased donor. 5 patients had a second renal transplant while 1 patient had a third renal transplant from a deceased donor as shown in Table 5.2.

Table 5.2: Showing the number of transplant and source of organ

Source of organ	Frequency	Percent
L1(First living)	11	23.4
T1(First deceased)	29	61.7
T2(Second deceased)	5	10.6
T3(Third deceased)	2	4.3
Total	47	100.0

5.1.1 Etiology of chronic renal failure:

Chronic glomerulonephritis 13 (27.7%) was the commonest cause of chronic renal failure leading to end stage renal disease. Chronic pyelonephritis was seen in 8 (17%) and adult polycystic kidney disease was seen in 5 (10.6%). All various other etiologies leading to chronic renal failure are detailed in Table 5.3.

Table 5.3: Etiology of chronic renal failure

Basic disease causing chronic renal failure	Frequency	Percent
ADPKD (Polycystic kidney disease)	5	10.6
CGN (Chronic glomerulonephritis)	13	27.7
CPN (Chronic pyelonephritis)	8	17.0
DRUG ABUSE	1	2.1
HYPERTENSION	5	10.6
IgA NEPHROPATHY	2	4.3
REFLUX NEPHROPATHY	4	8.5
SLE	1	2.1
TYPE1 Diabetes Mellitis	1	2.1
UNKNOWN	6	12.8
VASCULITIS	1	2.1
Total	47	100.0

5.1.2 Clinical indications for biopsy:

The most common indication for undergoing the renal biopsy procedure was renal dysfunction which was usually indicated by a rise in serum creatinine of at least 10%.

34 (72.3%) patients had renal dysfunction, 8 (17%) patients had delayed graft function (DGF), 4 (8.5%) had renal dysfunction associated with proteinuria and 1 patient underwent biopsy due to proteinuria without an associated renal dysfunction as shown in Table 5.4. Median time between the date of transplantation and biopsy was 157 days ± 275.7 (6-8878).

Table 5.4: Showing clinical indications for biopsy

Clinical reason for biopsy	Frequency	Percent
Rise in Creatinine	34	72.3
Rise in Creatinine+proteinuria	4	8.5
DGF	8	17.0
Proteinuria	1	2.1
Total	47	100.0

5.1.3 Histological diagnosis:

Histological diagnosis was available for all of the 47 allograft biopsies. Chronic allograft nephropathy (CAN) was seen in 14(29.8%), Acute Tubular Necrosis (ATN) was seen in 5(10.6%). Acute cellular rejection was seen in 19(40.4%) of which 7 were borderline, 9 Grade1a and 3 showed Grade 2a. Cyclosporine toxicity (CYA) was seen in 4(8.5%) and other various diagnosis are shown in Table 5.5. Banff 97 classification (Solez K, 2008) was used to score the grade of acute cellular rejection and chronic changes in all of the allograft biopsies as shown in Table 5.6.

Table 5.5: Histological diagnosis

DIAGNOSIS ON BIOPSY	NUMBER
Acute tubular necrosis (ATN)	5(10.6%)
Borderline Rejection	7(14.9%)
Chronic Allograft Nephropathy (CAN)	14(29.8%)
Cyclosporine toxicity (CYA)	4(8.5%)
Acute rejection (Grade1A)	9(19.1%)
Acute Rejection (Grade2A)	3(6.4%)
Haemolytic uremic syndrome (HUS)	1(2.1%)
IgA Nephropathy (IgA)	2(4.3%)
Normal	1(2.1%)
Post transplant lymphoproliferative disease (PTLD)	1(2.1)
Total	47

Descriptive variables are reported as percent

Table 5.6: Banff scoring of acute rejection and chronic changes

No of glomeruli	9.152 ±0.60
No of arteries	2.239±0.16
Glomerulitis (g)	0.130±0.05
Interstitial inflammation (i)	0.891±0.13
Tubulitis (t)	0.565±0.11
Intimal arteritis (v)	0.065±0.03
Glomerulopathy (cg)	0.108±0.06
Interstitial fibrosis (ci)	0.673±0.13
Tubular atrophy (ct)	0.587±0.13
Fibro-intimal thickening (cv)	0.326±0.09
Arteriolar hyalinosis (ah)	0.489±0.11
Mesangial matrix increase (mm)	0.260±0.07

Continuous variables are reported as mean ± standard error of means

5.1.4 Immunosuppression:

Most of the patients were on a combination therapy of various immunosuppessive drugs as per the protocol and clinical indication for each individual patient. Calcineurine inhibitors (Cyclosporine or Tacrolimus) based immunosuppression formed the basis of most of the immunosuppressive therapy (91.4%). Predisolone,

Mycophenolate Mofetil (MMF) and Cyclosporine (Neoral) was the most common combination, 9 (19.1%) and Neoral monotherapy was given seen in 9 (19.1%). Complete details of the rest are shown in Table 5.7.

Table 5.7: Immunosuppression therapy

Immunosuppression regime	Frequency	Percent
Azathioprine+Prednisolone	1	2.1
Azathioprine+Prednisolone+Neoral	2	4.3
MMF+Neoral	8	17.0
Neoral	9	19.1
Prednisolone+MMF	1	2.1
Prednisolone+MMF+Azathioprine	1	2.1
Prednisolone+MMF+Neoral	9	19.1
Prednisolone+Neoral	2	4.3
Rapamycin	1	2.1
Tacrolimus	1	2.1
Tacrolimus+MMF	8	17.0
Tacrolimus+MMF+,Prednisolone	3	6.4
Tacrolimus+Rapamycin+Prednisolone	1	2.1
Total	47	100.0

5.1.5: Analysis of graft biopsies for oxidative stress:

109 biopsy samples were analysed. The following proteins were detected:

Catalase kd-60 60 samples

MnSOD 25-kd 68 samples

CuZnSOD 23-kd 58 samples

Thioredoxin reductase 55 kd 58 samples

Thioredoxin(2b1) 12kd 51 samples

Thioredoxin kd62 21 samples

5.2 Western Blot results for oxidative stress markers from clinical biopsies:

Western blot analysis was done on several gels due to the large number of samples. On each gel samples were loaded from different clinical conditions. Beta actin was used as loading control.

For quantification purposes each gel had at least one sample from a chronic allograft nephropathy labelled as can which was used as a control as there was no adequate control biopsy. Donor biopsies were labelled as D, acute cellular rejection as acr, cyclosporine toxicity as cya and the biopsy reported as normal was labelled as n. Quantification graphs were produced as a percent change from chronic allograft nephropathy sample from each individual gel by performing densitometry. Data was analysed using Graphpad prism 5 software.

5.2.1 Gel 1:

Gel 1 was probed with various anti oxidant enzyme antibodies which were positive for MnSoD, CuZnSoD, Catalase and Thioredoxin reductase. For quantification purpose, one biopsy which was reported as normal, one biopsy with IgA nephropathy and 2 with CAN were grouped together and results were expressed as a percentage change compared to this group.

There were 2 biopsies from acute rejection and donor group. CuZnSoD density was higher in biopsies taken from acute cellular rejection and pre-implantation donor kidneys when compared to samples from CAN.

However only donor biopsy samples showed a significant increase ($p<0.05$) as shown in figure 5.1.

MnSod also was increased in both donors and acute cellular rejection but did not reach statistical significance.

Catalase and Thioredoxin reductase were also increased in acute cellular rejection and donor biopsies (p=not significant). Thioredoxin reductase was elevated in acute rejection compared to donor biopsy.

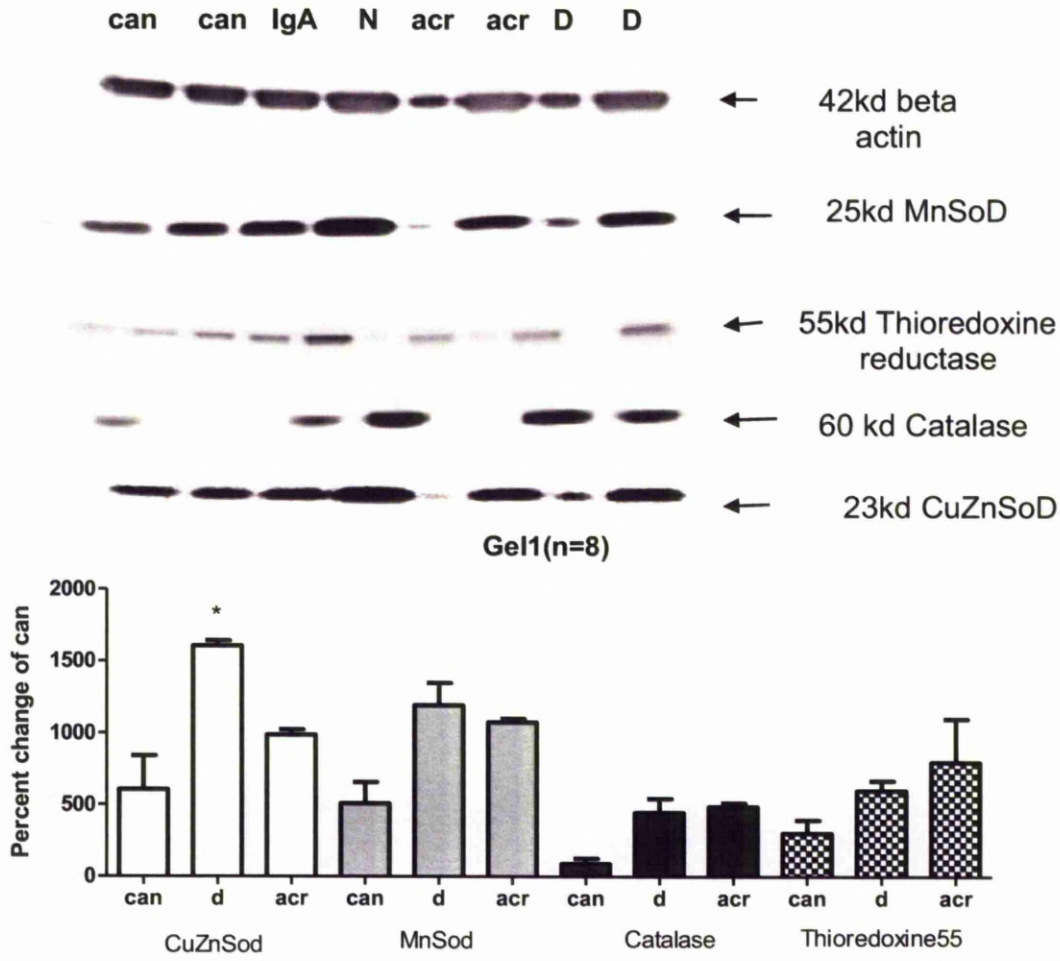


Figure 5.1:

Quantification of Gel1 showing CuZnSoD, MnSoD, Catalase & Thioredoxin 55

* P<0.05

5.2.2 Gel 2:

Western blot was positive for MnSoD, CuZnSod and Thioredoxin reductase55. Biopsies having acute injury like acute tubular necrosis and Haemolytic uremic syndrome were grouped together as ATN for quantification purpose.

There was a significant rise of MnSoD in donor samples and acute rejection samples (p<0.05) as shown in figure 5.2. Both CuZnSoD and Thioredoxin reductase 55 were

seen to be more in acute rejection, ATN and donors but not reaching statistical significance in any of them.

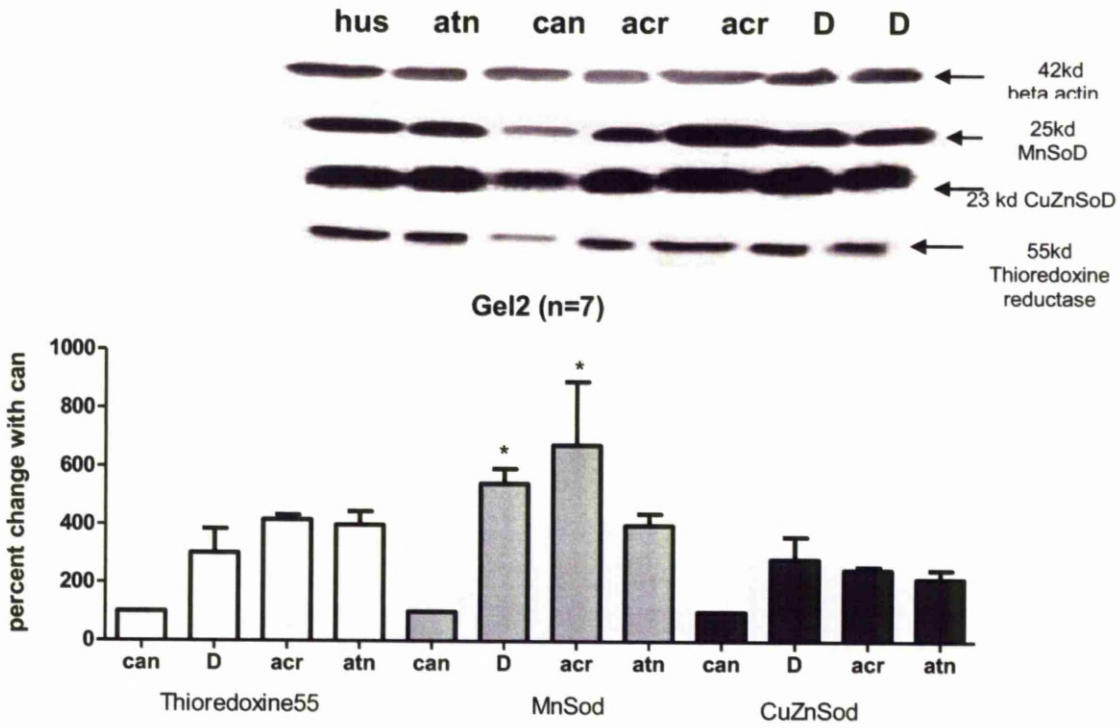


Figure 5.2: Quantification of Gel-2 showing MnSoD, CuZnSoD and Thioredoxin reductase

* P<0.05

5.2.3 Gel 3:

MnSod, CuZnSoD and thioredoxin reductase were positive. This gel had samples from cyclosporine toxicity (3), acute rejection (4), CAN (3). There were no samples from deceased donor kidneys or with ATN. MnSoD was increased in ACR and CYA group. Thioredoxin reductase 55 was also increased in ACR and CYA. CuZnSoD was also increased in ACR group but less than that seen in CYA as shown in Figure 5.3.

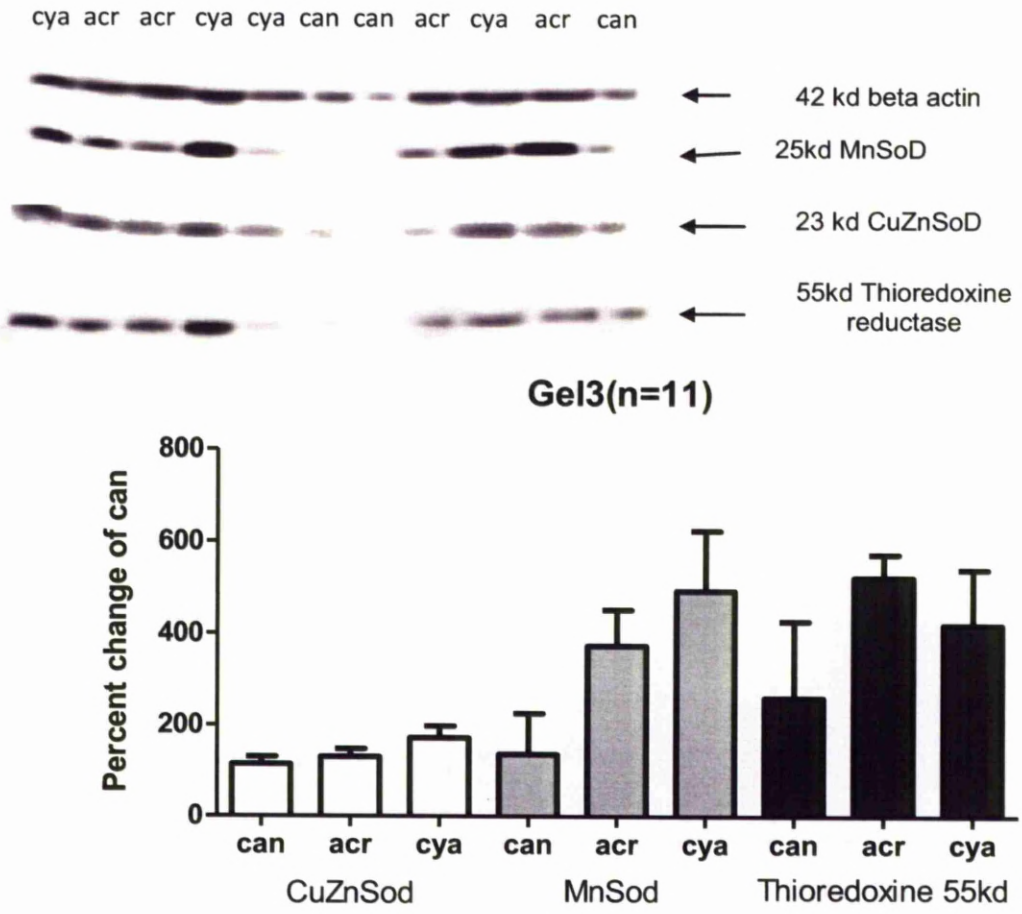


Figure 5.3: Quantification of Gel3 showing MnSod, CuZnSoD and Thioredoxin reductase

5.2.4 Gel4:

Catalase and thioredoxin12 were positive. There were 4 biopsies from CAN and 4 from ACR and 3 from CYA group. Catalase was slightly higher in CYA group but not

in ACR. Similarly Thioredoxin was also higher in CYA group but not in ACR as shown in figure

5.4.

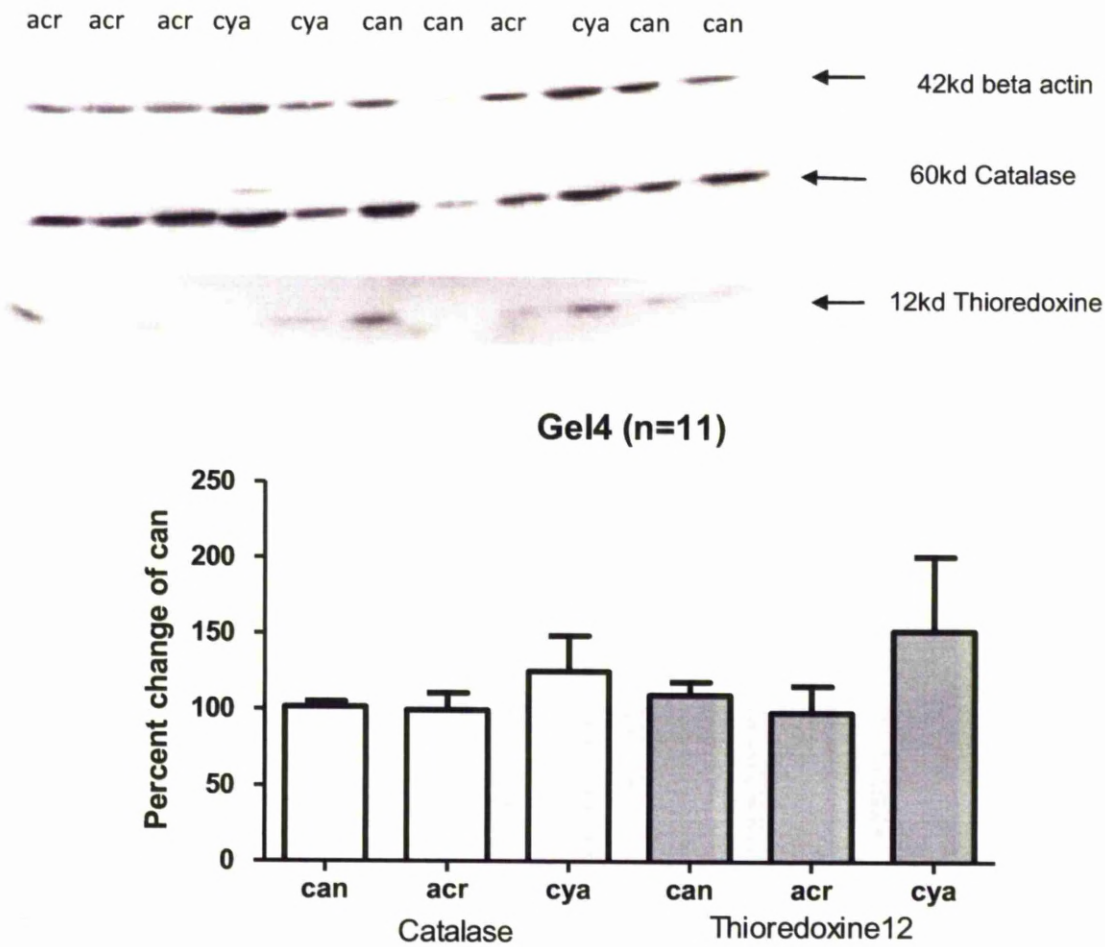


Figure 5.4: Quantification of Gel4 showing Catalase and Thioredoxin12

5.2.5 Gel 5:

MnSod, CuZnSoD and thioredoxin reductase were positive. There were 2 samples from CAN,6 samples from ACR,1 sample from donor and CYA each. For quantification the solitary CYA sample was grouped with ACR. MnSoD was increased in donor and ACR. CuZnSod was slightly higher in donor group but thioredoxin

reductase did not show any difference between the three groups as shown in figure 5.5.

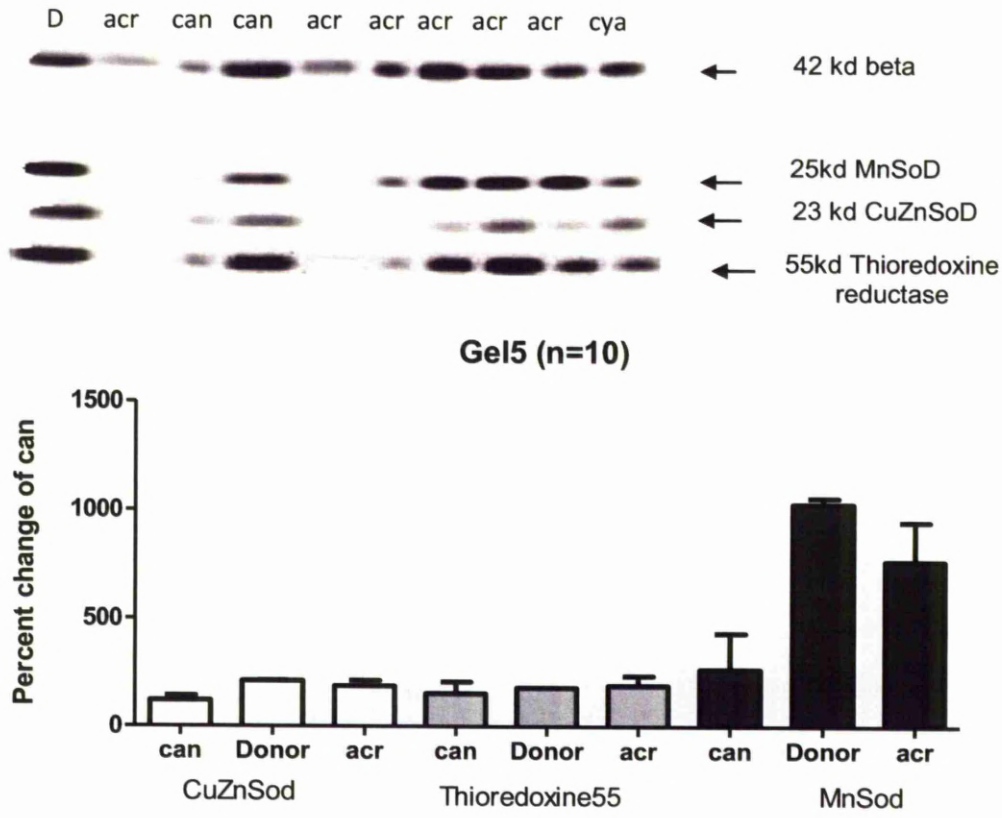


Figure 5.5: Quantification of Gel 5 showing MnSoD, CuZnSoD and Thioredoxin reductase 55

5.2.6 Gel 6:

Catalase and Thioredoxin 12 were positive. There were 2 biopsies from CAN and 5 from ACR, one sample from CYA and donor. For quantification CYA was grouped with ACR.

Catalase and thioredoxin did not show any significant change when compared with CAN as shown in figure 5.6.

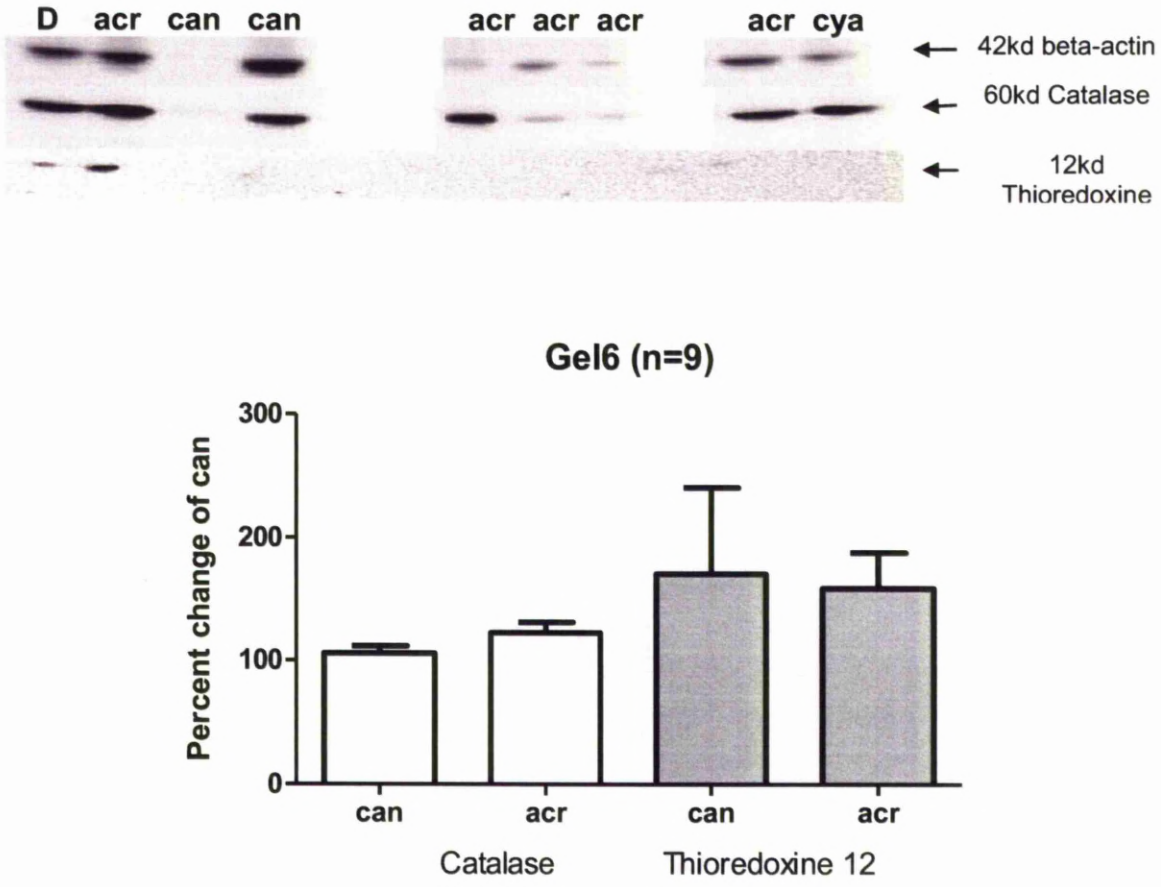


Figure 5.6: Quantification of Gel 6 showing catalase and thioredoxin 12

5.2.7 Gel 7:

Positive for catalase, MnSoD,CuZnSoD and thioredoxin reductase 55. There were 3 biopsies from donor group, 3 from CAN,2 from ATN and 2 from ACR. One biopsy from ATN also showed ACR. For quantification this was pooled with ATN.

Catalase was significantly higher in the donor group ($p<0.05$). CuZnSoD was also significantly higher in the donor group when compared to ACR and ATN groups but not when compared to the CAN group. Thioredoxin reductase was also significantly higher in donor group when compared to the ATN and ACR groups but not when

compared with CAN, similar to the pattern seen with CuZnSoD. Donor MnSod showed a significant rise when compared to all other group as shown in figure 5.7 ($p<0.05$).

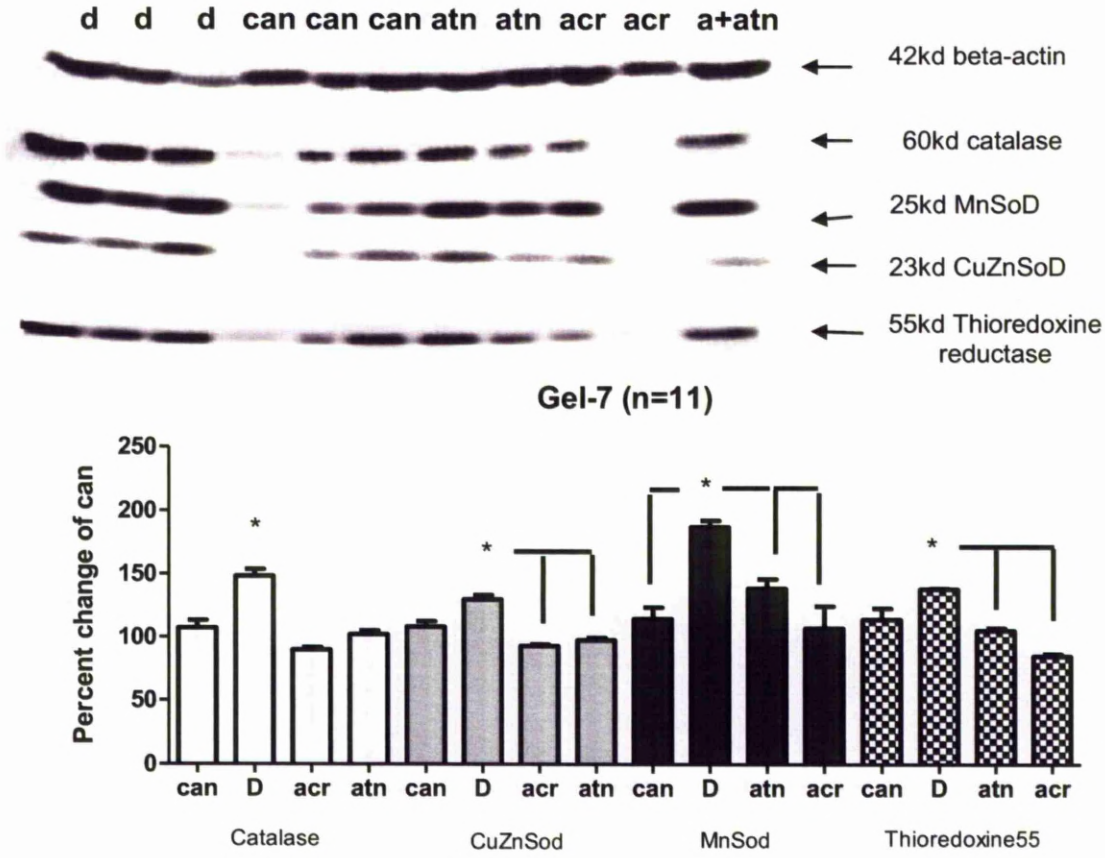


Figure 5.7: Quantification graph of Gel 7 showing MnSod, CuZnSoD, Catalase and Thioredoxin reductase 55

* $P<0.05$

5.2.8 Gel 8:

Positive for Catalase, CuZnSoD, MnSoD, thioredoxin reductase. 4 biopsies were in CAN group, 3 were from ACR, 3 from the donor group and one had HUS. HUS biopsy was excluded from quantitative analysis. MnSoD was increased in both the donor and ACR group when compared with CAN which was statistically significant between CAN and ACR only ($p<0.05$)

Catalase, CuZnSoD and Thioredoxin reductase all were slightly raised in Donor and ACR but not significantly as shown in figure 5.8.

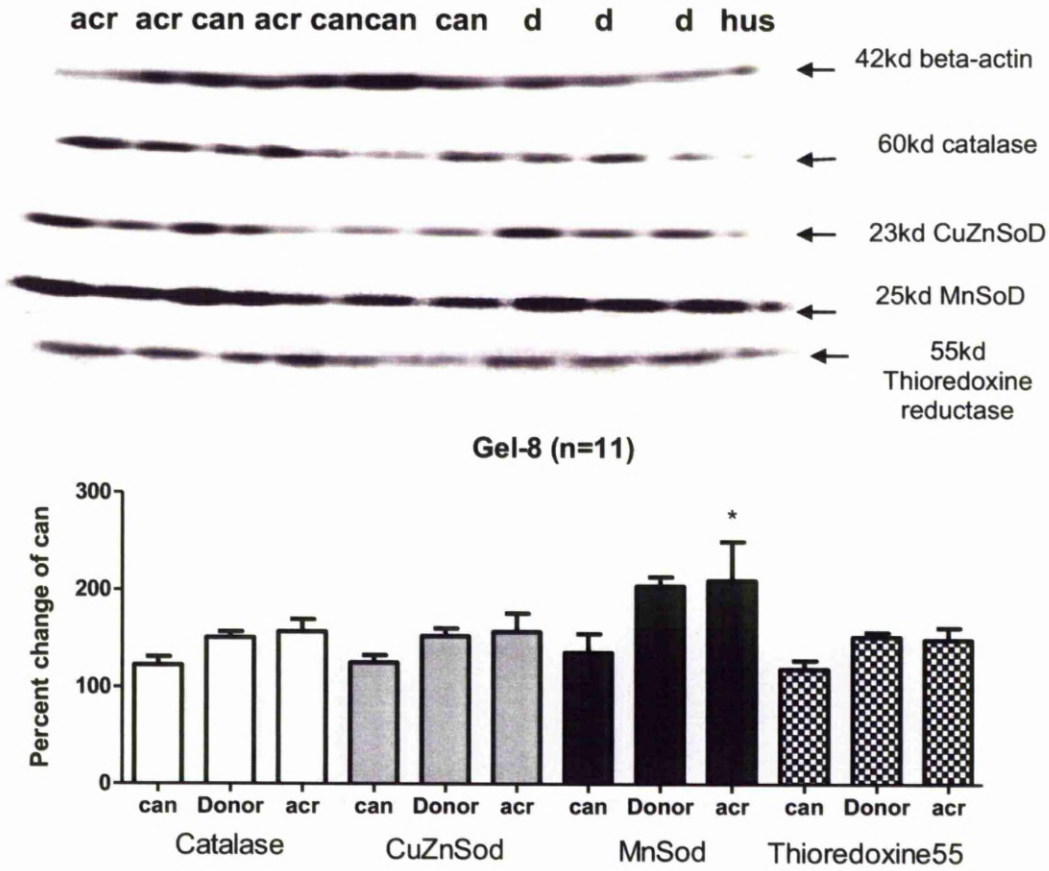


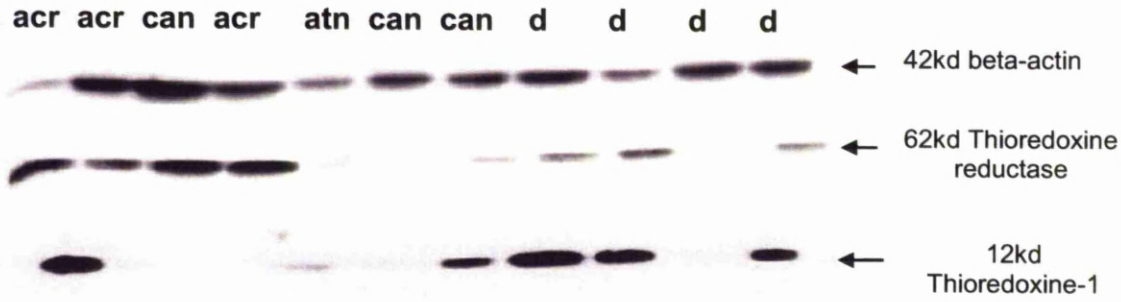
Figure 5.8: Quantification graph of Gel 8 showing Catalase, CuZnSoD, MnSoD, Thioredoxin55

* P<0.05

5.2.9 Gel 9:

Positive for Thioredoxin reductase 62 and thioredoxin 1. There were 4 samples from donors, 3 from CAN, 3 from ACR, and 1 from ATN. For quantification ATN was grouped with the donor group.

Thioredoxin 2b1 was increased in the donor and to a lesser extent in the ACR group. Thioredoxin reductase was increased in ACR and to a lesser extent in the donor group (p>0.05) as shown in figure 5.9.



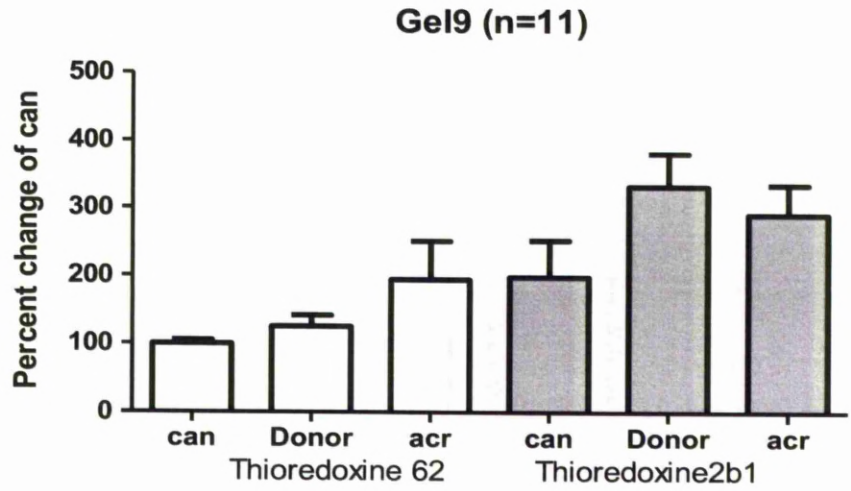
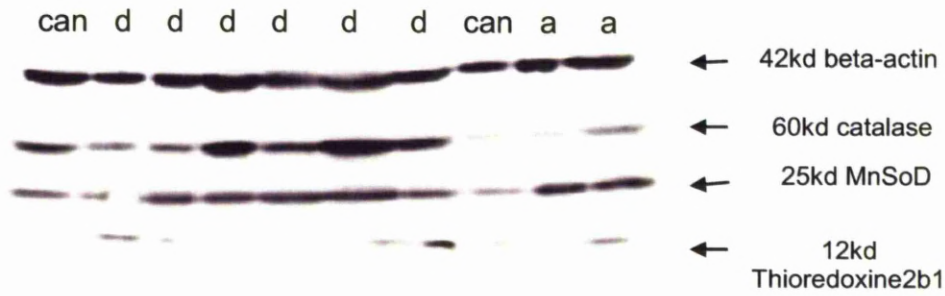


Figure 5.9: Quantification graph for Gel 9 showing Thioredoxin reductase 62kd and Thioredoxin 2b1

5.2.10 Gel 10:

Positive for Catalase, MnSoD, Thioredoxin 2b1.2 samples were from CAN, 6 from donors and 2 from ACR. MnSoD was higher in the donor group. Catalase and thioredoxin 2b1 did not show any difference when compared with the CAN group as shown in figure 5.10.



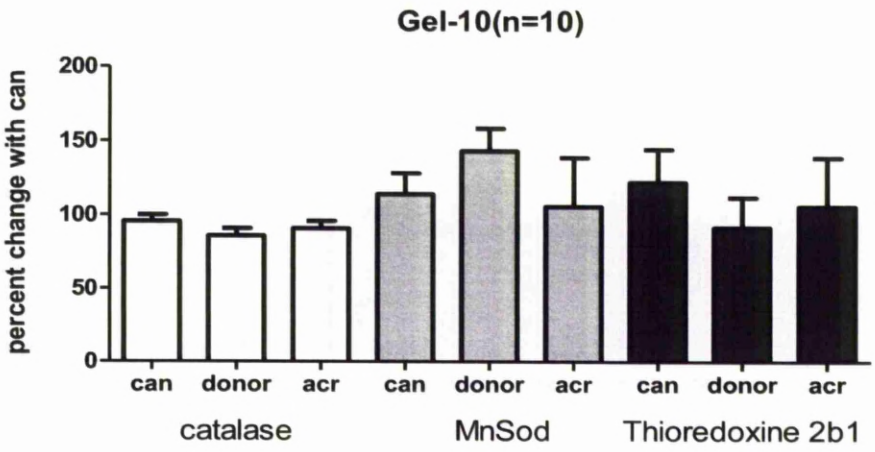
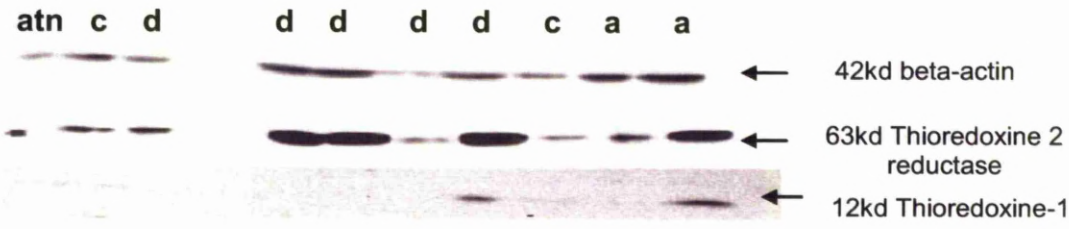


Figure 5.10: Quantification graph for Gel 10 showing Catalase, MnSoD and Thioredoxin 2b1

5.2.11 Gel 11:

Positive for Thioredoxin reductase 62 and thioredoxin 12. There were 2 samples from CAN, 5 donors, 2 ACR and 1 ATN. For quantification ATN was grouped with ACR. Thioredoxin 62 was higher in the donor group than in ACR P>0.05). Similarly thioredoxin1 was also higher in donor group but not significantly as shown in Figure 5.11.



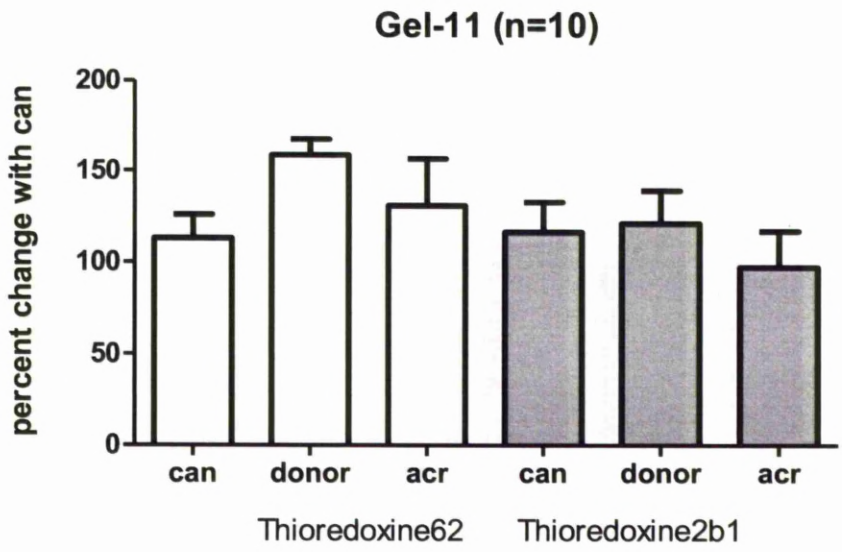


Figure 5.11: Quantification of Gel 11 showing thioredoxin 62 and thioredoxin 2b1

The results of all western blots were combined and plotted together. Mnsod, CuZnSod and Trx55 were increased in deceased donor, acute rejection and cyclosporine toxicity but did not reach statistical significance. All enzymes tested showed a reduced expression in chronic allograft nephropathy as shown in figure 5.12.

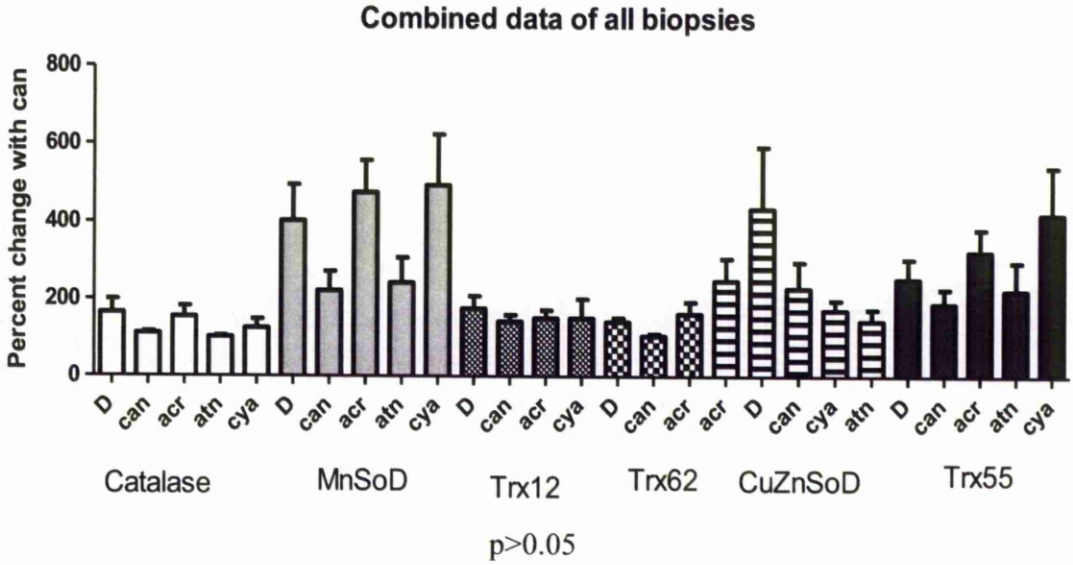


Figure 5.12: Comparison graph of all biopsy data

CHAPTER 5

DISCUSSION:

6. Chronic Kidney Disease:

Incidence and burden

Chronic kidney disease consistently affects 10–15% of the general population and the incidence of renal replacement therapy varies from around 100–120 per million of the population (Caskey et al, 2010). End stage kidney failure creates a huge health and economic burden to the nation with sufferers experiencing poorer than average health status and quality of life. Data from the UK Renal Registry shows that since 2000 there has been a 5% annual increase in prevalence of people requiring renal replacement therapy, amounting to 2% of the total National Health Service budget (Galliford and Game, 2009).

It has been known for several decades that transplantation is associated with both a survival and quality of life benefit for recipients when compared with age and health matched patients receiving other modalities of renal replacement therapy (Wolfe et al, 1999).

Health economics are increasingly important in modern healthcare provision. Renal transplantation can be heralded as an excellent example of healthcare technology that is cost saving and cost effective when compared to dialysis, and so the promotion of renal transplantation has an additional societal gain (Zelmer, 2007).

Increasing numbers of renal transplants are being performed. During 2009, 2600 kidney or kidney plus other organ transplants were performed in the UK, a 5% rise from the previous year (Webb et al, 2011).

6.1 DGF:

6.1.1 Incidence

Ischemia-reperfusion injury is an inevitable event accompanying kidney transplantation. It often leads to acute, ischemic tubular injury which manifests clinically as delayed graft function. The incidence of DGF is variable depending on the criteria used to define it. Halloran & Hunsicker reported a DGF rate of 20–29% in cadaver kidneys from the across the transplant centres in USA (Halloran and Hunsicker, 2001). Gavela Martinez et al from Spain have recently reported an incidence rate of 36.8% (Gavela Martinez et al, 2011).

The frequency of DGF in cadaver transplants has declined only slightly, from about 29 to 23% over the past decade. In contrast, acute rejection rates have fallen more steeply, and the fall in acute rejection has probably accounted for the improvement in survival. The incidence of DGF is about 6% in kidneys from living donors, higher than is generally realized (Halloran and Hunsicker, 2001).

6.1.2 Sequelae of DGF

Although the true long-term clinical importance of DGF has been debated, Yarlagadda et al published a meta-analysis showing DGF is associated with a 41% increased risk of graft loss at a mean 39,635 patient-years of follow-up and a 38% increased risk of acute rejection (Yarlagadda et al, 2009). Similarly Boom et al showed DGF influences long term renal function (Boom et al, 2001).

6.1.3 Pathophysiology of DGF:

The usual histological findings in DGF are of acute tubular necrosis (ATN), similar to ATN in native kidneys. Transplant ATN differs from native kidney ATN in having fewer tubular casts, occasional necrosis of complete tubular cross-sections, less tubular

dedifferentiation and regeneration, more calcium oxalate crystals, and more microcalcification and isometric vacuolization (possibly due to calcineurin inhibitor toxicity) (Olsen et al, 1989).

Cold ischemia and reperfusion during renal transplantation result in release of reactive oxygen species. Oxidative stress resulting from IRI has been shown by many studies to have a major role in causing DGF (Ahlenstiel et al, 2006).

In normal conditions, the body systems can easily detoxify the produced ROS with endogenous antioxidants such as SOD and CAT. However, if there is a pathological condition, like IRI, ROS is produced more than usual. A number of studies have demonstrated that higher or lower levels of endogenous antioxidants, decrease or increase the sensitivity to cell injury concomitantly (Markey et al, 1990; Lash and Tokarz, 1990). IRI has provoked wide interest and several studies have investigated IRI in several animal and cell culture models.

6.2 Simulated IRI and cell culture:

Creation of a simulated condition of oxidative stress was attempted within the constraints of a laboratory model. Human proximal tubular cells (HK-2) were chosen for the purpose of these experiments because of their proven track record, durability and predicted physiological response. Histochemical, immune cytochemical, and functional characteristics have shown that these cells reproduce experimental results.

IRI was simulated by incubating HK-2 cells with hydrogen peroxide. 0.5 mM of hydrogen peroxide was used for inducing anti-oxidant enzymes. Hydrogen peroxide when introduced exogenously is known to permeate through cell membranes and produce oxidative stress (Giorgio et al, 2007). High concentration of ROS cannot be detoxified to water by the intra-cellular antioxidant enzymes. Increased production of O_2^- leads to H_2O_2, and non-detoxified H_2O_2 reacts with Fe^{2+} (known as the Fenton

reaction) to produce •OH, which is the most toxic ROS to cellular organisms in the oxidative system.

Hydrogen peroxide is also a normal metabolite important for intra cellular signalling and is produced by SODs during the dismutation of superoxide anion (Halliwell and Gutteridge, 1984). It is also produced by TNF-α and cytokines to increase their own activities (Fiers, 1991).

Miguel et al have utilised hydrogen peroxide in a cell culture model of HeLa cells. They have shown acute and chronic treatment with hydrogen peroxide induced oxidative stress in a time and concentration dependent manner which was prevented with the use of anti-oxidants ascorbic acid and N-acetylcysteine (Miguel, Augusto, and Gurgueira, 2009).

Similarly Harrett et al also showed that prior exposure to sublethal H_2O_2 confirmed an adaptive response, resulting in a greater cellular resistance to subsequent toxic exposures. They also observed greater catalase, GPx, and CuZnSOD enzymatic activity and increased DNA protection (Jarrett and Boulton, 2005).

6.3 Antioxidant enzymes:

6.3.1 Glutathione peroxidase (GPx):

In these experiments HK-2 cells were subjected to exogenous oxidative stress by incubation with 0.5mM Hydrogen peroxide for 1 hour. They responded by showing an increased production of the antioxidant enzyme glutathione peroxidase. Glutathione peroxidase activity was increased in two phases one at 2 hrs and a bigger second peak at 24 hrs which was also statistically significant. GPx is an important enzymatic antioxidant enzyme. Cytosolic GPx is a selenoprotein that requires several secondary enzymes and co-factors (reduced glutathione, NADPH and glucose 6-phosphate) to function at high efficiency. There are also five GPx isoenzymes (de Haan et al, 1998).

Extra cellular GPx has been mainly shown to be produced in kidneys and HK-2 cells in cell cultures have been proven to be its source (Avissar et al, 1994), (Whitin et al, 2002). Several studies have reported involvement of GPx in oxidative stress in proximal tubules (van de Water, Zoeteweij and Nagelkerke, 1996; Zhu et al, 2008).

Sheldon et al have investigated the role of hydrogen peroxide in neonatal head injury, and showed GPx1 plays an important role in the defense against hydrogen peroxide and is thereby neuroprotective (Sheldon, Christen and Ferriero 2008).

Bose et al showed that an increased antioxidant defence through higher GSH content and greater of enzymes such as Cu-ZnSOD, catalase and GPx, provided protection from hydrogen peroxide in a cell culture model of a hamster cell line (Bose Girigoswami, Bhaumik and Ghosh, 2005).

Ng et al investigated the removal of hydrogen peroxide by GPx. The rate of removal of hydrogen peroxide increased with increasing GPx and they found that the rate of removal of hydrogen peroxide is affected by GPx and GSH (Ng et al, 2007).

6.3.2 Catalase:

In the present experiments with HK-2 cells, hydrogen peroxide failed to induce catalase activity. There could be several reasons for this but other studies have also shown catalase to be less responsive in experimental oxidative stress conditions and have postulated that it could be due to differential inhibition (Miguel, Augusto and Gurgueira 2009). Catalase is also reported to be inactivated by hydroxyl radicals while GPx and SOD are considerably less affected by these radicals (Pigeolet et al, 1990). Nitric oxide has also been shown to reduce catalase activity (Brown, 1995), (Raicevic et al, 2010).

The concentration of hydrogen peroxide used may also affect oxidative damage and micromolar concentrations of hydrogen peroxide (100 μM) may induce oxidative DNA damage more efficiently than millimolar concentrations (Nakamura, Purvis and

Swenberg, 2003). In this study millimolar concentrations were used for testing the anti-oxidant enzymes but micromolar concentrations were used for chemokine induction.

Cultured cells are also known to have reduced sensitivity when compared to intact renal tubular cells (Kim et al, 1998).

6.4 IL-8 (CXCL8) and Hydrogen peroxide:

In the second part of the model the induction of chemokines in response to oxidative stress was investigated. Basal secretion of IL-8 (CXCL8) was present in the supernatant which was higher in samples taken from serum free media. The basal secretion of IL-8 (CXCL8) is possibly the response of the cells to normal stress of growth inherent in cell culture system.

Bioprocess forces are known to be encountered during cell culture and include hydrodynamic shear caused by shaking of the flasks to aid their detachment following trypsinisation, forces produced during centrifugation prior to resuspending and shear stresses resulting from transfer through capillaries or by pipetting to resuspend cell pellets (Veraitch et al, 2008).

Recent evidence has shown that bioprocess forces produced during capillary transfer or pipetting of cell suspensions can enhance osteogenic responses and are considered to be harmful to cells (Brindley et al, 2011).

These experiments showed that IL-8 (CXCL8) as measured by ELISA increased after incubation with 30 and 300 μM of hydrogen peroxide in a dose dependent manner. IL-1β alone significantly increased IL-8 (CXCL8) more than with hydrogen peroxide alone. The IL-1β and 300μM hydrogen peroxide combination induced IL-8 (CXCL8) more than the combination of 30μM H_2O_2 and IL-1β showing a synergy between IL-1β and hydrogen peroxide.

Hydrogen peroxide is known to increase IL-8 (CXCL8) production in a variety of cells. Similar to our results of an increase in IL-8 (CXCL8) after hydrogen peroxide treatment, Andreucci et al also found an increase in IL-8 (CXCL8) after hydrogen peroxide stimulation in HK-2 cells (Andreucci et al, 2009). Shimada et al showed a similar rise in IL-8 (CXCL8) from gastric epithelial cells when stimulated with hydrogen peroxide (Shimada et al, 1999).

DeForge et al also showed the stimulatory effect of hydrogen peroxide on IL-8 (CXCL8) production by HepG2 cells, A549 pulmonary type II epithelial cells, and human skin fibroblasts (DeForge et al, 1993).

6.4.1 Intracellular signal pathways

ROS, apart from altering the oxidative mechanisms also cause activation of the intra-cellular pathways that cause inflammation. NF-κB is a critical signalling molecule in H_2O_2 induced inflammation and in responses produced by a variety of stimuli that include growth factors, lymphokines, ultraviolet irradiation, pharmacological agents, and oxidant stress. NF-κB is maintained as a latent form in the cytoplasm of cells where it is complexed to I-κB inhibitor proteins; NF-κB activation involves the phosphorylation and subsequent degradation of the inhibitory protein I-κB as well as the phosphorylation of p65 protein. Thus, released NF-κB dimer can translocate into the nucleus. In the nucleus, NFκB binds with consensus sequences of various genes, activating their transcription (Gloire, Legrand-Poels, and Piette, 2006).

Similarly Enesa et al showed the mechanism for the pro-inflammatory effects of H_2O_2, prolonged the nuclear localization of NF-κB in activated cells by suppressing the negative regulatory functions of IκBα (Enesa et al, 2008).

Some authors have also linked activation of JNK pathway to be up-regulated by ROS (Kunduzova et al, 2002). Fernandes et al showed the similar role of several signaling

pathways, such as NF-κB, p38 and MAPK in oxidative stress-induced up-regulation of IL-8 (CXCL8) in retinal cells (Fernandes et al, 2008).

Kina et al showed Fe^{2}+ mediated ERK pathway activation as an important signal transduction pathway in ROS-induced IL-8 secretion in epithelial cells (Kina et al, 2009).

Expression of chemokines, such as IL-8(CXCL8), which attracts granulocytes into the graft, thereby causing further damage to the tissues as they themselves produce more ROS and chemokines. Infiltrating inflammatory cells may also release transforming growth factor (TGF-β1), platelet-derived growth factor and fibroblast growth factor-2 which stimulate resident fibroblast cells to proliferate and release excessive extracellular matrix (Furuichi et al, 2008; Araki et al, 2006).

An increase of interleukin-8 mRNA expression after I/R both in syngeneic and allogeneic transplantation was associated with a marked infiltration of granulocytes in renal tissue. A CXCR2 inhibitor, repertaxin, was effective in preventing granulocyte infiltration and renal function impairment (Cugini et al, 2005).

6.4.2 Hydrogen peroxide and IL-1β (synergism) :

Cytokines like IL-1 beta, TNF or the bacterial product LPS in both a time- and dose-dependent manner are known to increase expression of IL-8 (CXCL8) mRNA and secreting IL-8 (CXCL8) peptide (Schmouder et al, 1992).

Ivison et al proved the link and mechanism of hydrogen peroxide mediated IL-8 (CXCL8) induction. This was dose dependant and associated with synergistic phosphorylation of p38 MAP kinase and with prolonged I-κB degradation and NF-κB activation (Ivison et al, 2010).

Li et al have also showed that redox regulation of NIK by hydrogen peroxide is mechanistically important in IL-1beta induction of NFκB activation (Li and Engelhardt, 2006). Enesa et al observed that 100 μM H_2O_2 combined with cytokines (IL-1β, TNFα)

induced IL-8 (CXCL8) in synergy in cultured epithelial cells. They also observed that the capacity of H_2O_2 to enhance IL-8 (CXCL8) induction in response to TNFα was suppressed in cultures that were pretreated with the anti-oxidant *N*-acetyl-cysteine 10 mM (Enesa et al, 2008).

H_2O_2 had a significant positive modulatory effect a synergism on the activation of NF-κB by TNF-α (de Oliveira-Marques et al, 2007).

6.4.3 Inhibitory role of hydrogen peroxide:

In these experiments involving both IL-8 (CXCL8) and MCP-1 (CCL2), inhibition of both was seen when a high dose of hydrogen peroxide, 300 μM, was used in combination with IL-1β. Higher levels of oxidative exposure can turn a potential positive stimulus by H_2O_2 into an inhibitory effect (de Oliveira-Marques et al, 2007) and this maybe the reason we detected significant inhibition when high dose of hydrogen peroxide was used with NAC.

H_2O_2 has a fine-tuning regulatory role, comprising both a pro-inflammatory control loop that increases pathogen removal and an anti-inflammatory control loop, which avoids an exacerbated harmful inflammatory response (de Oliveira-Marques et al, 2007).

6.5 MCP-1 (CCL2)

These experiments showed MCP-1 (CCL2) could be induced with IL-1β alone and in combination with 300μM hydrogen peroxide potentiated this response. There was a synergistic response when IL-1β and 300μM hydrogen peroxide were combined. NAC could partially suppress the induction of both IL-1β alone and combination of IL-1β with hydrogen peroxide. MCP-1 (CCL2) is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages. Migration of monocytes from the blood stream across the vascular endothelium is required for routine immunological

surveillance of tissues, as well as in response to inflammation (Deshmane et al, 2009).

HK-2 cells have been shown to express MCP-1 (CCL2) mRNA and protein. The mRNA expression increases with exposure to calcium oxalate crystals. Catalase and superoxide dismutase reduces MCP-1 (CCL2) expression suggesting the involvement of ROS in its regulation (Habibzadegah-Tari, Byer and Khan, 2006).

ROS such as superoxide and hydrogen peroxide have also been shown to mediate TNF-α induced MCP-1 (CCL2) expression in endothelial cells (Chen et al, 2004). Elevated MCP-1 (CCL2) expression might be responsible for increased monocyte infiltration in the injured kidney. Sung et al demonstrated enhanced MCP-1 (CCL2) expression in rat kidney during ischemia - reperfusion injury and that it is mediated by NF-κB activation and oxidative stress (Sung et al, 2002).

Inhibition of MCP-1 (CCL2) signalling in ischemic renal injury have shown to have a protective role (Furuichi et al, 2003). Takaya et al have shown that ERK signalling is involved in BSA-induced MCP-1 (CCL2) expression in mProx cells (Takaya et al, 2003). Takahashi et al have also suggested JNK- and IκB-dependent pathways regulate MCP-1 (CCL2) synthesis and also found NAC could suppress MCP-1 (CCL2) production (Takahashi et al, 2008).

NAC has been shown to reduce the expression of MCP-1 (CCL2) in a rat model of acute pancreatitis (Xu, Wu and Shen, 2008).

6.6 NAC:

NAC has been known to have mucolytic and antioxidant properties. Beneficial effects of N-acetyl-cysteine on renal injury triggered by ischemia and reperfusion have been shown by Di Giono (Di Giorno et al, 2006).

NAC has also been shown to protect kidneys against oxidative damage, reversed oxidant responses and protected rat renal proximal tubules from in vitro simulated reperfusion injury (Sehirli et al, 2003).

Antonicelli et al investigated the effect of Nacystelyn, a recently developed lysine salt of NAC upon interleukin IL-8 (CXCL8) release and the activation of the redox-sensitive transcription factors AP-1, NF-kappaB, and C/EBP in a human alveolar epithelial cell line (A549). Nacystelyn at 5 mM enhanced intracellular glutathione (GSH) after 4 h and abolished hydrogen peroxide induced IL-8 (CXCL8) release in A549 cells. This was associated with inhibition of NF-κB. (Antonicelli et al, 2002)

Ayvaz et al have reported the benefit of NAC in intestinal ischemic injury as well as, reperfusion in rats (Ayvaz et al, 2009). NAC in combination with Erdosteine has also been shown to improve the biochemical results of IR injury (Erdogan et al, 2006).

Nitescu et al have shown NAC improves kidney function, and reduces renal interstitial inflammation, in rats subjected to renal IRI. These effects were associated with increased renal glutathione levels, and decreased plasma ascorbyl concentrations, suggesting that NAC attenuates renal and systemic oxidative stress in this model (Nitescu et al, 2006).

Jiang et al have shown NAC inhibits the induction IL-8 (CXCL8) gene and protein by LPS via suppression of NF-κB activation in human uterine smooth cells (Jiang et al, 2008).

Wuyts et al have shown in human airway smooth muscle cells, adding NAC decreased IL-17 induced IL-8 (CXCL8) production. The clinical significance of these in vitro findings for prevention or treatment of chronic rejection after lung transplantation remains to be investigated (Wuyts et al, 2004).

Zhang et al have proposed that NAC exerts its protective effect in part by directly scavenging ROS and in part via ERK1/2 activation. In their study NAC could protect

HK-2 cells against the toxic effects of a hydroquinone metabolite (Zhang, Lau and Monks, 2011).

Ueno et al have recently showed the role of NAC in controlling oxidative stress against osteoblasts and have suggested further in vivo studies to test its therapeutic potential as a local antioxidative stress drug (Ueno et al, 2011).

6.6.1 Clinical use of NAC: Despite many favourable results in the lab models, NAC has not been used widely in the clinical world. So far it has shown some clinical benefit in preventing DGF and contrast nephropathy.

6.6.2 Prevention of DGF:

Danilovic et al have shown clinical benefit of NAC in renal transplant recipients. Use of NAC resulted in reduced rate of DGF and better renal function was seen for up to one year (Danilovic et al, 2011).

NAC administration has also showed an improvement in the histopathological findings of ischemia/reperfusion damages (Ayvaz et al, 2009). Donor pre treatment with NAC has been shown to preserve renal metabolism and improve outcomes of I/R injured kidney transplants (Fuller et al, 2004). Fuentes et al have shown NAC treatment in patients with stable renal function after transplantation increased high-density lipoprotein cholesterol and antioxidant molecules in relation to glutathione peroxidase, with a positive influence on renal function (Ruiz Fuentes et al, 2008).

6.6.3 Prevention of contrast nephropathy:

Prophylactic oral administration of NAC, along with hydration, prevents the reduction in renal function induced by iopromide, a nonionic, low-osmolality contrast agent, in

patients with chronic renal insufficiency (Tepel et al, 2000), (Shalansky et al, 2005; Birck et al, 2003). Briguori et al have reported that NAC was better than fenoldopam mesylate to prevent contrast agent-associated nephrotoxicity (Briguori et al, 2004).

6.7 Cold storage:

Kidneys are kept in cold storage prior to implantation for variable time periods. This period is crucial in limiting the ischemic damage. The preservative fluids used during this time provide a unique therapeutic window where anti oxidants like NAC can be utilised to prevent and control IRI.

Several research groups have made significant advances in this area by testing a range of compounds as additives to preservation solutions to improve cellular or tissue function during cold storage or after transplantation. Some reports suggest that the addition of bioflavonoids and trophic factor supplementation to preservation solutions should be explored further because these compounds prevented lipid peroxidation, mitochondrial dysfunction, and loss of cell viability during cold storage of porcine and canine renal tubular cells (Ahlenstiel et al, 2006; Kwon et al, 2007).

Salhudeen et al have shown cold storage modulated the expression of intrinsic antioxidants, particularly that of mitochondrial Mn-SOD, and addition of extrinsic antioxidants to University of Wisconsin solution markedly enhanced its ability to protect cells against cold-induced structural and functional injury (Salahudeen, Joshi and Jenkins, 2001).

The addition of the antioxidant deferoxamine to the University of Wisconsin (UW) preservation solution has been shown to improve glomerular filtration rate and decrease cell death in a syngeneic rat kidney transplant model (Huang et al, 2003).

Adding mitoquinone, a mitochondria-targeted antioxidant, to University of Wisconsin preservation solution could offer protection against cold storage injury (Mitchell et al, 2011).

Cold storage slows down metabolic reactions to preserve organ quality while allowing time for recipient selection and transport. Although this procedure is extremely valuable, cold storage has been shown to cause vasoconstriction, tubular and endothelial injury, and cell death which can result in discardment of kidneys. Fortifying the storage solution with deferoxamine or preconditioning the donor kidneys with hemeoxygenase-1 may prove viable clinical strategies to limit cold ischemic injury (Salahudeen, 2004).

6.8 Oxidative stress markers in renal biopsies:

This experimental work on renal allograft biopsies was aimed to determine the oxidative stress occurring after transplantation. Donor pre-implantation biopsies provided a unique opportunity to detect oxidative stress occurring due to IRI and thus link up with the IRI in a cell culture model.

Renal allograft biopsies are routinely performed to evaluate renal dysfunction. The procedure generally has a low complication rate. The following complications have been reported: gross hematuria 3.5%, perirenal hematomas 2.5%, arterio-venous fistulas 7.3% and vasovagal reactions 0.5%. Major complications requiring invasive procedures such as blood transfusions or urinary catheterisation are seen in 1% of cases (Schwarz et al, 2005).

Some authors have advocated renal biopsies for prognostic purpose. Schwarz et al have shown that morphological changes of CAN expressed as tubular atrophy and interstitial fibrosis precedes the decline of renal functional (Schwarz et al, 2005). Other

authors have suggested its use to detect subclinical rejection (Thierry et al, 2011), (Henderson, Nankivell and Chapman, 2011).

A total of 61 biopsies were analysed and the following oxidative stress enzymes were detected by western blot: Catalase 60kd, MnSOD 25kd, CuZnSOD 23kd, Thioredoxin reductase 55 kd Thioredoxin (2b1) 12kd, and Thioredoxin 62kd.

Upregulation of most of the anti-oxidant enzymes was detected in biopsies obtained from deceased donor kidneys prior to transplantation. MnSoD was expressed more in donor kidneys, acute rejection and cyclosporine toxicity. CuZnSoD was also elevated in donor and acute rejection biopsies. Catalase was elevated in donor and acute rejection biopsy samples. Thioredoxin was elevated in donor biopsies while thioredoxin reductases were elevated in donors, acute rejection and cyclosporine toxicity. Ischemic injury and related oxidative stress is possibly the reason for the elevation in these enzymes. However, due to lack of adequate control biopsies the results were difficult to interpret.

6.8.1 Catalase:

Catalase has been investigated by several authors in biopsy material and Reuter et al conducted proteome analysis of rat renal allografts and found that the expression of catalase was downregulated (Reuter et al, 2010). Dutkiewicz also found that polymorphism in the catalase gene is associated with DGF in kidney allograft recipients (Dutkiewicz et al, 2010).

Granqvist et al showed that antioxidative defence was higher in the tubulointerstitial compartment than in the glomerular cells and found a reduction in glomerular enzymes catalase and glutathione peroxidase-3, and -4 in nephritic kidneys. The tubular gene expression was downregulated for catalase, glutathione peroxidase-3, and thioredoxin reductase-1 and -2 (Granqvist et al, 2010).

Cadaveric kidneys when compared to live donor kidneys have exhibited a distinctly different set of anti oxidant genes originating from the tubulointerstitial compartment (Kainz et al, 2004). The deceased donor kidneys have long periods of cold storage resulting in hypoxic damage and thus oxidative stress. Our results from such kidneys confirm this phenomenon as biopsies taken from these kidneys showed an increase in oxidative stress enzymes like MnSod, CuZnSod, catalase and the thioredoxin system. Donor kidneys from recipients with impaired allograft function also showed activation of genes mainly belonging to the functional classes of immunity, signal transduction, and oxidative stress response (Kainz et al, 2007).

6.8.2 SOD:

CuZnSOD comprises ~90% of total SOD activity in a eukaryotic cell (Liu et al, 2004). Mahmoud et al have also reported an increase in CuZnSoD from cattle liver biopsies after inducing oxidative stress (Abd Ellah et al, 2009).

Salahudeen et al have shown that genetic expression of mitochondrial MnSOD, but not of cytosolic CuZnSOD or of glutathione peroxidase, increased with cold exposure, suggesting mitochondria as a cellular source of free radicals and activation under cold injury (Salahudeen et al, 2000).

MnSoD over expression in renal tubular cells has been shown to protect against high-glucose-induced oxidative stress (Munusamy and MacMillan-Crow, 2009). Chen et al showed increased mRNA levels of CuZnSOD and MnSOD enzymes in the cortex and medulla of rats subjected to hypoxia (Chen et al, 2003).

Saba et al have investigated the role in cold preservation of MnSoD and mitochondrial injury. Short term cold I/R resulted in inactivation of MnSOD. These data suggest that compounds designed to prevent early mitochondrial injury in kidneys that undergo

cold preservation would significantly improve renal function and graft survival following transplantation (Saba, Munusamy and Macmillan-Crow, 2008).

Loss of mRNA of catalase and GPX may be the first markers of alterations in cellular redox in ischemia-reperfusion injury. The mRNA for MnSOD was upregulated at all time points of ischemia-reperfusion injury suggest that antioxidant genes are not coordinately expressed during ischemia-reperfusion and that the differential loss of antioxidant enzymes may be the contributing factor(s) towards the heterogeneous renal tissue damage as a result of ischemia-reperfusion induced oxidative stress (Dobashi et al, 2000).

Some authors have also suggested that mitochondrial dysfunction is an early event in a rat model of allotransplant and may play a causative role in the development of chronic allograft nephropathy (MacMillan-Crow et al, 2001).

6.8.3Thioredoxin system:

Thioredoxin reductase has been reported to be prominently expressed in the proximal tubules of rodent kidneys (Rundlof et al, 2000). Kasuno et al reported secretion of thioredoxin from proximal tubules into urine during renal ischemia/reperfusion and suggested it may have a protective effect against renal ischemia/reperfusion injury (Kasuno et al, 2003). Conterato et al have also found elevated thioredoxin reductase activity after exposure to lead acetate in rat kidneys along with other anti- oxidant enzymes like catalase and superoxide dismutase (Conterato et al, 2007).

In proximal tubules nuclear and luminal localization was detected for Trx1 after IRI. The cytosolic TrxR1 was detected in all tubular segments, especially strong in distal convoluted tubules and thin segments of the inner medulla. Trx2 was diffusely expressed in all regions of the kidney. After IRI, Trx2 immunoreactivity increased in the mTAL segments and in the lumen of thin segments in the inner medulla .TrxR2,

which was primarily detected in connective tissue in the sham group, showed a segment-specific increase after IRI, most notably in the lumina and epithelial cells of distal convoluted tubules.

Strong immunoreactivity for TrxR2 in luminal compartments was also observed in the inner medulla (Godoy et al, 2011).

6.8.4 Cyclosporine nephrotoxicity:

Several of the biopsy specimens studied were taken from patients with cyclosporine nephrotoxicity. Histology is characterised by tubular vacuolation and an ATN like picture. Results from western blot assay revealed that all of the anti oxidant enzymes tested were elevated in cyclosporine nephrotoxicity. Thioredoxin reductase in particular showed the maximum elevation although other enzymes like MnSoD, CuZnSoD, Catalase and Thioredoxin 12kda were also elevated.

Cyclosporine nephrotoxicity is manifested by renal insufficiency due to glomerular disease and abnormalities in tubular function. Several mechanisms have been proposed for cyclosporine A-induced nephrotoxicity, such as sodium retention, renal vasoconstriction, renal hypoxia, as a consequence of renal vasoconstriction, stimulation of the renin-angiotensin system, activation of the sympathetic nervous system, impaired synthesis of nitric oxide, and increased growth factor-B1 (Atessahin, Ceribasi and Yilmaz, 2007; Lee et al, 2001; Sanchez-Pozos et al, 2010).

Oxidative stress has been implicated in cyclosporine nephrotoxicity. Increase in ROS generation and lipid peroxidation has been shown to affect renal function and interstitial fibrosis (Burdmann et al, 2003; Atasoyu et al, 2006).

Buffoli et al have reported reduction of both oxidative stress and increased iNOS and NF-kB expression induced by Cyclosporine with use of red wine polyphenol (Buffoli et al, 2005).

NAC treatment significantly protected animals against cyclosporine induced structural and functional impairment of kidneys implicating the role of oxidative stress in the pathogenesis of CsA-induced nephrotoxicity (Tariq et al, 1999).

Magendramani et al have shown that cyclosporine nephrotoxicity is mediated through increased expressions of inducible nitric oxide synthase (iNOS), NF-*k*B and matrix metalloproteinase-2 which was altered by an anti-oxidant *S*-allylcysteine thereby rendering protection to the kidney cells (Magendiramani et al, 2009).

6.9 Limited clinical benefit of anti oxidants:

It is generally thought that reactive oxygen species (ROS) are involved in a wide variety of diseases, including ischemia-reperfusion, cancer and various types of inflammation. Despite this widespread involvement of ROS there is a lack of widespread use of anti oxidants in clinical use.

N-acetylcysteine, despite the growing evidence of benefit, has very limited used only for treating over-doses of acetaminophen and for improvement of bronchial mucous fluidity which might affect thiol proteases (Jones, 1998).

Edaravone has been used for the treatment of acute cerebral infarctions (Edaravone Acute Infarction Study Group, 2003), (Houkin et al, 1998).

Recombinant Cu, Zn-SOD and Mn-SOD have been produced, but their plasma half-lives are very short, limiting their use (Somack, Saifer and Williams, 1991).

In a porcine model anti oxidants used including NAC failed to show any benefit (Kuntscher et al, 2007). Although the therapeutic use of antioxidants is now widespread, some of these have been shown to be successful, but many appear to have no or little benefit, in terms of being beneficial to general health or in disease prevention (Suzuki, 2009).

Many anti-oxidants show strong positive effects in the laboratory, but only a few of these drugs are used as anti-oxidants in humans. Some plausible reasons for this are that because ROS production occurs ubiquitously in aerobic cells and chemical reactivity of ROS takes place in a non-specific manner; to find statistically significant differences in different cells or tissues is difficult.

Human blood and tissues already contain an abundance of anti-oxidants. Moreover, many anti-oxidative enzymes are readily induced by many cytokines and stresses. A preconditioning phenomenon in ischemia-reperfusion could be due to the production of inducible antioxidative enzymes, such as MnSOD, while xanthine oxidase is induced which is a potential source of ROS. Because of this, when anti-oxidative drugs are administered to humans, their clinical benefits are frequently difficult to verify (Suzuki, 2009). Also even if expression of enzyme or protein is seen it may not necessarily result in an increase in activity (Weydert and Cullen, 2010).

In clinical terms Kubal et al did not find any correlation between hypoxia times and mitochondrial complex activities and DGF (Kubal et al, 2009).

6.10 Future directions:

This study has clearly shown that HK-2 cells are responsive to various physiological stimuli like hydrogen peroxide and cytokines. Oxidative stress is possibly involved in the various responses seen. N-Acetyl cysteine was able to block these responses effectively further reinforces the importance of oxidative stress. The challenge remains to reproduce these results in clinical situations and perhaps further similar studies in vivo model will be beneficial. We had used high dosage of NAC (10mMol), this dosage maybe difficult to use for clinical purposes so further studies with reduced dosage titration of NAC is suggested. High dosage of NAC can be delivered in cold perfusion fluids.

The cell culture model has proven to be successful in studying oxidative stress and cyclosporine is another agent which could be studied further here. The clinical biopsies have also shown an increase in oxidative stress in cyclosporine toxicity group.

The renal allograft biopsies also showed us that oxidative stress was involved in various acute injuries occurring within the allograft. However due to lack of control biopsies the results could not be interpreted effectively. Future studies involving renal allograft biopsies must include a control sample from normal kidney.

Acute injuries like cold ischemia in donor biopsies were associated with oxidative stress further correlation with warm and cold ischemia times are recommended which may pave the way for therapeutic use of NAC.

6.11 Conclusions:

DGF is an important clinical outcome after kidney transplantation, and one that needs to be addressed by funding agencies, trialists and clinicians. DGF is both an outcome of a renal allograft and a predictor for its subsequent course. In an era of a tremendous shortage of kidneys for transplantation, every effort should be made to improve the survival of the transplanted kidneys in the recipient. Therefore, it is imperative that we implement strategies to reduce the incidence of DGF in an effort to improve long-term graft survival.

Elucidation of the pathophysiology of renal ischaemia and reperfusion injury has contributed to the development of strategies to decrease the rate of delayed graft function, focusing on donor management, organ procurement and preservation techniques, and pharmacological agents (vasodilators, antioxidants, anti-inflammatory agents). Several new drugs show promise in animal studies in preventing or

ameliorating ischaemia-reperfusion injury and possibly delayed graft function, but definitive clinical trials are lacking.

Because ROS production is very common and the human body has an abundant capacity for producing anti-oxidants, it is difficult to obtain statistical differences in trials designed to evaluate an anti-oxidative drug. Additional resources will be needed, if we are to use anti-oxidants in human clinical settings, e.g. drug delivery to a specific region, appropriate setting of evaluation goals in clinical trials, new approaches to understanding the details of anti-oxidative systems.

In spite of these drawbacks, the additional use of anti-oxidants in clinical settings is clearly warranted, and could contribute positively to human health. It is generally thought that ROS are involved in a wide variety of diseases, including ischemia-reperfusion, cancer and various types of inflammation.

Histological evaluations before transplantation may enable the identification of organs unsuitable for single implantation (Navarro et al, 2011) but such evaluation of oxidative stress enzymes is not done in routine practice.

Based on the 2009 Organ Procurement and Transplantation Network/Scientific Registry of Transplant Recipients Annual Report, 16% of kidneys recovered from potential deceased donors were discarded because of cold ischemia times, biopsy findings, or the inability to locate a recipient (Klein et al, 2010).

IRI is now acknowledged as a key target to improve graft outcome, particularly through ex-vivo interventions during transport. This phase is indeed an underused therapeutic window, although few studies have highlighted its importance. The present results show that NAC is a drug with potential for altering the oxidative stress and chemokine induction thus reducing the cellular injury during ischemia reperfusion injury. This potent drug warrants further investigation.

References

Abd Ellah, Mahmoud R., et al. 2009. Superoxide dismutase activity as a measure of hepatic oxidative stress in cattle following ethionine administration. *The Veterinary Journal* 182, no. 2:336-341.

Ahlenstiel, T., G. Burkhardt, H. Kohler, and M. K. Kuhlmann. 2006. Improved cold preservation of kidney tubular cells by means of adding bioflavonoids to organ preservation solutions. *Transplantation* 81, no. 2:231-239.

Ali, F., and S. Sultana. 2011. Repeated short-term stress synergizes the ROS signalling through up regulation of NFkB and iNOS expression induced due to combined exposure of trichloroethylene and UVB rays. *Molecular and cellular biochemistry*.

Alkhatib, G., et al. 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science (New York, N.Y.)* 272, no. 5270:1955-1958.

Andreucci, M., et al. 2009. Downregulation of cell survival signalling pathways and increased cell damage in hydrogen peroxide-treated human renal proximal tubular cells by alpha-erythropoietin. *Cell proliferation*.

Appay, Victor, and Sarah L. Rowland-Jones. 2001. RANTES: a versatile and controversial chemokine. *Trends in immunology* 22, no. 2:83-87.

Arai, R. J., et al. 2006. Nitric oxide induces thioredoxin-1 nuclear translocation: possible association with the p21Ras survival pathway. *Biochemical and biophysical research communications* 348, no. 4:1254-1260.

Arstall, Margaret A., et al. 1995. N-Acetylcysteine in Combination With Nitroglycerin and Streptokinase for the Treatment of Evolving Acute Myocardial Infarction : Safety and Biochemical Effects. *Circulation* 92, no. 10:2855-2862.

Atasoyu, E. M., et al. 2006. Investigation of the effect of hyperbaric oxygen on experimental cyclosporine nephrotoxicity. *Basic & clinical pharmacology & toxicology* 98, no. 2:150-154.

Atessahin, A., A. O. Ceribasi, and S. Yilmaz. 2007. Lycopene, a carotenoid, attenuates cyclosporine-induced renal dysfunction and oxidative stress in rats. *Basic & clinical pharmacology & toxicology* 100, no. 6:372-376.

Audard, V., et al. 2008. Renal transplantation from extended criteria cadaveric donors: problems and perspectives overview. *Transplant international : official journal of the European Society for Organ Transplantation* 21, no. 1:11-17.

Avissar, N., et al. 1994. Human kidney proximal tubules are the main source of plasma glutathione peroxidase. *The American Journal of Physiology* 266, no. 2 Pt 1:C367-75.

Ayvaz, S., et al. 2009. The effects of N-acetylcysteine on intestinal ischemia/reperfusion injury in rats. *Saudi medical journal* 30, no. 1:24-29.

Baggiolini, M., A. Walz, and S. L. Kunkel. 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *The Journal of clinical investigation* 84, no. 4:1045-1049.

Banerjee, A., V. Pirrone, B. Wigdahl, and M. R. Nonnemacher. 2011. Transcriptional regulation of the chemokine co-receptor CCR5 by the cAMP/PKA/CREB pathway. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 65, no. 4:293-297.

Barrientos, A., et al. 1994. Glomerular hyperfiltration as a nonimmunologic mechanism of progression of chronic renal rejection. *Transplantation* 57, no. 5:753-756.

Beckman, J. S., and W. H. Koppenol. 1996. Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *American Journal of Physiology - Cell Physiology* 271, no. 5 40-5:.

Beers, R. F.,Jr, and I. W. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *The Journal of biological chemistry* 195, no. 1:133-140.

Bennett, L. D., J. M. Fox, and N. Signoret. 2011. Mechanisms regulating chemokine receptor activity. *Immunology* 134, no. 3:246-256.

Berndt, Carsten, Christopher H. Lillig, and Arne Holmgren. 2007. Thiol-based mechanisms of the thioredoxin and glutaredoxin systems: implications for diseases in the cardiovascular system. *AJP - Heart and Circulatory Physiology* 292, no. 3:H1227-1236.

Bindoli, Alberto, et al. 2009. Thioredoxin reductase: A target for gold compounds acting as potential anticancer drugs. *Coordination Chemistry Reviews* 253, no. 11-12:1692-1707.

Birck, R., et al. 2003. Acetylcysteine for prevention of contrast nephropathy: Meta-analysis. *Lancet* 362, no. 9384:598-603.

Bjornstedt, M., et al. 1994. The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. *The Journal of biological chemistry* 269, no. 47:29382-29384.

Bladh, L. G., et al. 2005. Identification of target genes involved in the antiproliferative effect of glucocorticoids reveals a role for nuclear factor-(kappa)B repression. *Molecular endocrinology (Baltimore, Md.)* 19, no. 3:632-643.

Boom, H., et al. 2001. Delayed graft function influences renal function but not survival. *Transplantation proceedings* 33, no. 1-2:1291.

Borgnia, M., S. Nielsen, A. Engel, and P. Agre. 1999. Cellular and molecular biology of the aquaporin water channels. *Annual Review of Biochemistry* 68, 425-458.

Boros, P., and J. S. Bromberg. 2006. New cellular and molecular immune pathways in ischemia/reperfusion injury. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 6, no. 4:652-658.

Bose Girigoswami, K., G. Bhaumik, and R. Ghosh. 2005. Induced resistance in cells exposed to repeated low doses of H2O2 involves enhanced activity of antioxidant enzymes. *Cell biology international* 29, no. 9:761-767.

Boys, J. A., et al. 2010. Effects of dantrolene on ischemia-reperfusion injury in animal models: a review of outcomes in heart, brain, liver, and kidney. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research* 58, no. 7:875-882.

Brasier, A. R. 2006. The NF-kappaB regulatory network. *Cardiovascular toxicology* 6, no. 2:111-130.

Briguori, C., et al. 2004. N-Acetylcysteine versus fenoldopam mesylate to prevent contrast agent-associated nephrotoxicity. *Journal of the American College of Cardiology* 44, no. 4:762-765.

Brindley, D., et al. 2011. Bioprocess forces and their impact on cell behavior: implications for bone regeneration therapy. *Journal of tissue engineering* 2011, 620247.

Brown, G. C. 1995. Reversible binding and inhibition of catalase by nitric oxide. *European journal of biochemistry / FEBS* 232, no. 1:188-191.

Buffoli, B., et al. 2005. Provinol prevents CsA-induced nephrotoxicity by reducing reactive oxygen species, iNOS, and NF-kB expression. *The journal of*

histochemistry and cytochemistry : official journal of the Histochemistry Society 53, no. 12:1459-1468.

Burdmann, Emmanuel A., Takeshi F. Andoh, Luis Yu, and William M. Bennett. 2003. Cyclosporine nephrotoxicity. *Seminars in nephrology* 23, no. 5:465-476.

Caskey, Fergus J., et al. 2010. The EVEREST study: an international collaboration*. *NDT Plus* 3, no. 1:28-36.

Cerra, F. B., T. Z. Lajos, M. Montes, and J. H. Siegel. 1975. Hemorrhagic infarction: A reperfusion injury following prolonged myocardial ischemic anoxia. *Surgery* 78, no. 1:95-104.

Chang, J. X., et al. 2005. Functional and morphological changes of the gut barrier during the restitution process after hemorrhagic shock. *World journal of gastroenterology : WJG* 11, no. 35:5485-5491.

C Chapple in Chapter Genitourinary system, Applied basic science for basic surgical training ed AT Raftery page 535 Churchill Livingstone 2000

Chapman, Jeremy R., Philip J. O'Connell, and Brian J. Nankivell. 2005. Chronic Renal Allograft Dysfunction. *Journal of the American Society of Nephrology* 16, no. 10:3015-3026.

Chen, C. F., S. Y. Tsai, M. C. Ma, and M. S. Wu. 2003. Hypoxic preconditioning enhances renal superoxide dismutase levels in rats. *The Journal of physiology* 552, no. Pt 2:561-569.

Chen, J., A. Edwards, and A. T. Layton. 2010. Effects of pH and medullary blood flow on oxygen transport and sodium reabsorption in the rat outer medulla. *American journal of physiology.Renal physiology* 298, no. 6:F1369-83.

Chen, Q., et al. 2007. Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion. *American Journal of Physiology - Cell Physiology* 292, no. 1:.

Chen, X. L., Q. Zhang, R. Zhao, and R. M. Medford. 2004. Superoxide, H2O2, and iron are required for TNF-alpha-induced MCP-1 gene expression in endothelial cells: role of Rac1 and NADPH oxidase. *American journal of physiology.Heart and circulatory physiology* 286, no. 3:H1001-7.

Claiborne, A. *CRC Handbook of Methods for Oxygen Radical Research*; CRC Press: Boca Raton, FL, USA, 1985.

Conterato, G. M., et al. 2007. Effect of lead acetate on cytosolic thioredoxin reductase activity and oxidative stress parameters in rat kidneys. *Basic & clinical pharmacology & toxicology* 101, no. 2:96-100.

Cugini, D., et al. 2005. Inhibition of the chemokine receptor CXCR2 prevents kidney graft function deterioration due to ischemia/reperfusion. *Kidney international* 67, no. 5:1753-1761.

Daemen, M. A., et al. 2001. Apoptosis and chemokine induction after renal ischemia-reperfusion. *Transplantation* 71, no. 7:1007-1011.

Daly, P. J., et al. 2009. The single insult of hypoxic preconditioning induces an antiapoptotic response in human proximal tubular cells, in vitro, across cold storage. *BJU international* 103, no. 2:254-259.

Danilovic, A., et al. 2011. Protective Effect of N-acetylcysteine on Early Outcomes of Deceased Renal Transplantation. *Transplantation proceedings* 43, no. 5:1443-1449.

Das, K. C., Y. Lewis-Molock, and C. W. White. 1997. Elevation of manganese superoxide dismutase gene expression by thioredoxin. *American journal of respiratory cell and molecular biology* 17, no. 6:713-726.

de Haan, J. B., et al. 1998. Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the

oxidative stress-inducing agents paraquat and hydrogen peroxide. *The Journal of biological chemistry* 273, no. 35:22528-22536.

de Oliveira-Marques, V., L. Cyrne, H. S. Marinho, and F. Antunes. 2007. A quantitative study of NF-kappaB activation by H2O2: relevance in inflammation and synergy with TNF-alpha. *Journal of immunology (Baltimore, Md.: 1950)* 178, no. 6:3893-3902.

de Vries, B., et al. 2004. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *The American journal of pathology* 165, no. 5:1677-1688.

DeForge, L. E., et al. 1993. Regulation of interleukin 8 gene expression by oxidant stress. *The Journal of biological chemistry* 268, no. 34:25568-25576.

Deshmane, S. L., S. Kremlev, S. Amini, and B. E. Sawaya. 2009. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research*.

Di Giorno, C., et al. 2006. Beneficial effect of N-acetyl-cysteine on renal injury triggered by ischemia and reperfusion. *Transplantation proceedings* 38, no. 9:2774-2776.

di Mari, J. F., R. Davis, and R. L. Safirstein. 1999. MAPK activation determines renal epithelial cell survival during oxidative injury. *The American Journal of Physiology* 277, no. 2 Pt 2:F195-203.

Dobashi, K., et al. 2000. Kidney ischemia-reperfusion: modulation of antioxidant defenses. *Molecular and cellular biochemistry* 205, no. 1-2:1-11.

Dreher, I., C. Schmutzler, F. Jakob, and J. Kohrle. 1997. Expression of selenoproteins in various rat and human tissues and cell lines. *Journal of trace elements in*

medicine and biology : organ of the Society for Minerals and Trace Elements (GMS) 11, no. 2:83-91.

Du, Y. T., W. B. Dong, M. Y. Wang, and Y. L. Hang. 2005. Replication of a model of injury to human renal proximal tubular cells induced by hypoxia/reoxygenation. *Zhongguo wei zhong bing ji jiu yi xue = Chinese critical care medicine = Zhongguo weizhongbing jijiuyixue* 17, no. 10:619-622.

Dunbar, Lisa A., and Michael J. Caplan. 2001. Ion Pumps in Polarized Cells: Sorting and Regulation of the Na+,K+- and H+,K+-ATPases. *Journal of Biological Chemistry* 276, no. 32:29617-29620.

Dutkiewicz, G., et al. 2010. The association of -262C/T polymorphism in the catalase gene and delayed graft function of kidney allografts. *Nephrology (Carlton, Vic.)* 15, no. 5:587-591.

Edaravone Acute Infarction Study Group. 2003. Effect of a novel free radical scavenger, edaravone (MCI-186), on acute brain infarction. Randomized, placebo-controlled, double-blind study at multicenters. *Cerebrovascular diseases (Basel, Switzerland)* 15, no. 3:222-229.

Enesa, K., et al. 2008. Hydrogen peroxide prolongs nuclear localization of NF-kappaB in activated cells by suppressing negative regulatory mechanisms. *The Journal of biological chemistry* 283, no. 27:18582-18590.

Epp, O., R. Ladenstein, and A. Wendel. 1983. The refined structure of the selenoenzyme glutathione peroxidase at 0.2-nm resolution. *European journal of biochemistry / FEBS* 133, no. 1:51-69.

Erdogan, H., et al. 2006. Protein oxidation and lipid peroxidation after renal ischemia-reperfusion injury: protective effects of erdosteine and N-acetylcysteine. *Urological research* 34, no. 1:41-46.

Fiers, W. 1991. Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. *FEBS letters* 285, no. 2:199-212.

Finkel, T., and N. J. Holbrook. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* 408, no. 6809:239-247.

Flohe, L., and W. A. Gunzler. 1984. Assays of glutathione peroxidase. *Methods in enzymology* 105, 114-121.

Frei, B., L. England, and B. N. Ames. 1989. Ascorbate is an outstanding antioxidant in human blood plasma. *Proceedings of the National Academy of Sciences of the United States of America* 86, no. 16:6377-6381.

Fuller, T. F., N. Serkova, C. U. Niemann, and C. E. Freise. 2004. Influence of donor pretreatment with N-acetylcysteine on ischemia/reperfusion injury in rat kidney grafts. *The Journal of urology* 171, no. 3:1296-1300.

Furuichi, K., et al. 2003. Gene therapy expressing amino-terminal truncated monocyte chemoattractant protein-1 prevents renal ischemia-reperfusion injury. *Journal of the American Society of Nephrology : JASN* 14, no. 4:1066-1071.

Furuichi, K., T. Wada, S. Kaneko, and P. M. Murphy. 2008. Roles of chemokines in renal ischemia/reperfusion injury. *Frontiers in bioscience : a journal and virtual library* 13, 4021-4028.

Galliford, J., and D. S. Game. 2009a. Modern renal transplantation: present challenges and future prospects. *Postgraduate medical journal* 85, no. 1000:91-101.

Ganong WF ed in Review of medicl physiology Chapter38 Renal function and micturition

page 675, 20th edition Mc Graw Hill publishers 2001

Gavela Martinez, E., et al. 2011. Delayed graft function after renal transplantation: an unresolved problem. *Transplantation proceedings* 43, no. 6:2171-2173.

Gigante, M., et al. 2011. Molecular and genetic basis of inherited nephrotic syndrome. *International journal of nephrology* 2011, 792195.

Gilmore, T. D. 2006. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* 25, no. 51:6680-6684.

Giral-Classe, M., et al. 1998. Delayed graft function of more than six days strongly decreases long-term survival of transplanted kidneys. *Kidney international* 54, no. 3:972-978.

Godoy, J. R., et al. 2011. Segment-specific overexpression of redoxins after renal ischemia and reperfusion: protective roles of glutaredoxin 2, peroxiredoxin 3, and peroxiredoxin 6. *Free radical biology & medicine* 51, no. 2:552-561.

Gourishankar, Sita, and Philip F. Halloran. 2002. Late deterioration of organ transplants: a problem in injury and homeostasis. *Current opinion in immunology* 14, no. 5:576-583.

Granqvist, A., et al. 2010. Impaired glomerular and tubular antioxidative defense mechanisms in nephrotic syndrome. *American journal of physiology.Renal physiology* 299, no. 4:F898-904.

Gutteridge, J. M. 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical chemistry* 41, no. 12 Pt 2:1819-1828.

Habibzadegah-Tari, P., K. G. Byer, and S. R. Khan. 2006. Reactive oxygen species mediated calcium oxalate crystal-induced expression of MCP-1 in HK-2 cells. *Urological research* 34, no. 1:26-36.

Haddad, J. J. 2002. Redox regulation of pro-inflammatory cytokines and IkappaB-alpha/NF-kappaB nuclear translocation and activation. *Biochemical and biophysical research communications* 296, no. 4:847-856.

Halliwell, B. 1999. Antioxidant defence mechanisms: from the beginning to the end (of the beginning). *Free radical research* 31, no. 4:261-272.

Halliwell, B., and J. M. Gutteridge. 1995. The definition and measurement of antioxidants in biological systems. *Free radical biology & medicine* 18, no. 1:125-126.

Halliwell, B., and J. M. Gutteridge. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *The Biochemical journal* 219, no. 1:1-14.

Halloran, P. F., and L. G. Hunsicker. 2001. Delayed Graft Function: State of the Art, November 10-11, 2000. Summit Meeting, Scottsdale, Arizona, USA. *American Journal of Transplantation* 1, no. 2:115-120.

Hansen, J. M., H. Zhang, and D. P. Jones. 2006. Mitochondrial thioredoxin-2 has a key role in determining tumor necrosis factor-alpha-induced reactive oxygen species generation, NF-kappaB activation, and apoptosis. *Toxicological sciences : an official journal of the Society of Toxicology* 91, no. 2:643-650.

Hassanain, M., et al. 2009. Delayed graft function has an equally bad impact on deceased donor renal graft survival in both standard criteria donors and expanded criteria donors. *Transplantation proceedings* 41, no. 1:133-134.

Henderson, L. K., B. J. Nankivell, and J. R. Chapman. 2011. Surveillance protocol kidney transplant biopsies: their evolving role in clinical practice. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 11, no. 8:1570-1575.

Hoffmann, E., O. Dittrich-Breiholz, H. Holtmann, and M. Kracht. 2002. Multiple control of interleukin-8 gene expression. *Journal of leukocyte biology* 72, no. 5:847-855.

Holtmann, H., et al. 1999. Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *Molecular and cellular biology* 19, no. 10:6742-6753.

Hornick, P. 2006. Direct and indirect allorecognition. *Methods in molecular biology (Clifton, N.J.)* 333, 145-156.

Houkin, K., et al. 1998. Neuroprotective effect of the free radical scavenger MCI-186 in patients with cerebral infarction: clinical evaluation using magnetic resonance imaging and spectroscopy. *Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association* 7, no. 5:315-322.

Hoy, W. E., et al. 2003. A stereological study of glomerular number and volume: preliminary findings in a multiracial study of kidneys at autopsy. *Kidney international.Supplement* (83), no. 83:S31-7.

Huang, H., Z. He, L. J. Roberts 2nd, and A. K. Salahudeen. 2003. Deferoxamine reduces cold-ischemic renal injury in a syngeneic kidney transplant model. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 3, no. 12:1531-1537.

Huang, Y., H. Rabb, and K. L. Womer. 2007. Ischemia-reperfusion and immediate T cell responses. *Cellular immunology* 248, no. 1:4-11.

Hussein, A. A., Z. H. El-Daken, N. Barakat, and H. Abol-Enein. 2011. Renal Ischemia/Reperfusion Injury: Possible Role of Aquaporins. *Acta physiologica (Oxford, England)*.

Inci, I., et al. 2007. N-acetylcysteine attenuates lung ischemia-reperfusion injury after lung transplantation. *The Annals of Thoracic Surgery* 84, no. 1:240-6; discussion 246.

Isowa, N., et al. 2000. Human thioredoxin attenuates hypoxia-reoxygenation injury of murine endothelial cells in a thiol-free condition. *Journal of cellular physiology* 182, no. 1:33-40.

Jacobs, M. D., and S. C. Harrison. 1998. Structure of an IkappaBalpha/NF-kappaB complex. *Cell* 95, no. 6:749-758.

Jarrett, S. G., and M. E. Boulton. 2005. Antioxidant up-regulation and increased nuclear DNA protection play key roles in adaptation to oxidative stress in epithelial cells. *Free radical biology & medicine* 38, no. 10:1382-1391.

Jones, A. L. 1998. Mechanism of action and value of N-acetylcysteine in the treatment of early and late acetaminophen poisoning: a critical review. *Journal of toxicology.Clinical toxicology* 36, no. 4:277-285.

Jones, D. P. 2006. Redefining oxidative stress. *Antioxidants & redox signaling* 8, no. 9-10:1865-1879.

Jushinskis, J., et al. 2009. Risk factors for the development of delayed graft function in deceased donor renal transplants. *Transplantation proceedings* 41, no. 2:746-748.

Kainz, A., et al. 2004. Alterations in gene expression in cadaveric vs. live donor kidneys suggest impaired tubular counterbalance of oxidative stress at implantation. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 4, no. 10:1595-1604.

Kainz, A., et al. 2007. Gene-expression profiles and age of donor kidney biopsies obtained before transplantation distinguish medium term graft function. *Transplantation* 83, no. 8:1048-1054.

Kanwar, Y. S., A. Linker, and M. G. Farquhar. 1980. Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. *The Journal of cell biology* 86, no. 2:688-693.

Karin, M. 1999. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. *Oncogene* 18, no. 49:6867-6874.

Karnovsky, M. J., and S. K. Ainsworth. 1972. The structural basis of glomerular filtration. *Advances in Nephrology from the Necker Hospital* 2, 35-60.

Kasuno, K., et al. 2003. Protective roles of thioredoxin, a redox-regulating protein, in renal ischemia/reperfusion injury. *Kidney international* 64, no. 4:1273-1282.

Kim, Jae-Sung, Yingai Jin, and John J. Lemasters. 2006. Reactive oxygen species, but not Ca2+ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. *AJP - Heart and Circulatory Physiology* 290, no. 5:H2024-2034.

Kirkman, H. N., M. Rolfo, A. M. Ferraris, and G. F. Gaetani. 1999. Mechanisms of protection of catalase by NADPH. Kinetics and stoichiometry. *The Journal of biological chemistry* 274, no. 20:13908-13914.

Klein, A. S., et al. 2010. Organ donation and utilization in the United States, 1999-2008. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 10, no. 4 Pt 2:973-986.

Kondo, N., H. Nakamura, H. Masutani, and J. Yodoi. 2006. Redox regulation of human thioredoxin network. *Antioxidants & redox signaling* 8, no. 9-10:1881-1890.

Kosieradzki, M., and W. Rowinski. 2008. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. *Transplantation proceedings* 40, no. 10:3279-3288.

Kubal, C. A., et al. 2009. Mitochondrial complex activity in donor renal grafts, cold ischemia time, and recovery of graft function. *Transplantation* 87, no. 7:1037-1039.

Kumar, C. K., N. Yanagawa, A. Ortiz, and H. M. Said. 1998. Mechanism and regulation of riboflavin uptake by human renal proximal tubule epithelial cell line HK-2. *The American Journal of Physiology* 274, no. 1 Pt 2:F104-10.

Kuntscher, V., et al. 2007. Does the administration of antioxidants as scavengers of reactive oxygen species in kidney transplantation really have sense? *Bratislavske lekarske listy* 108, no. 9:385-387.

Kusuoka, H., M. C. Camilion de Hurtado, and E. Marban. 1993. Role of sodium/calcium exchange in the mechanism of myocardial stunning: Protective effect of reperfusion with high sodium solution. *Journal of the American College of Cardiology* 21, no. 1:240-248.

Kwon, Y. S., J. D. Foley, C. J. Murphy, and J. F. McAnulty. 2007. The effect of trophic factor supplementation on cold ischemia-induced early apoptotic changes. *Transplantation* 83, no. 1:91-94.

Lakshminarayanan, V., D. W. Beno, R. H. Costa, and K. A. Roebuck. 1997. Differential regulation of interleukin-8 and intercellular adhesion molecule-1 by H2O2 and tumor necrosis factor-alpha in endothelial and epithelial cells. *The Journal of biological chemistry* 272, no. 52:32910-32918.

Land, W. G. 2011. Emerging role of innate immunity in organ transplantation Part I: evolution of innate immunity and oxidative allograft injury. *Transplantation reviews (Orlando, Fla.)*.

Lappas, M., M. Permezel, and G. E. Rice. 2003. N-Acetyl-cysteine inhibits phospholipid metabolism, proinflammatory cytokine release, protease activity, and nuclear factor-kappaB deoxyribonucleic acid-binding activity in human fetal membranes in vitro. *The Journal of clinical endocrinology and metabolism* 88, no. 4:1723-1729.

Larsen, C. G., et al. 1989. The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science (New York, N.Y.)* 243, no. 4897:1464-1466.

Lee, J. A., and D. G. Allen. 1992. Changes in intracellular free calcium concentration during long exposures to simulated ischemia in isolated mammalian ventricular muscle. *Circulation research* 71, no. 1:58-69.

Lee, Suk H., et al. 2001. Effect of iron and ascorbate on cyclosporine-induced oxidative damage of kidney mitochondria and microsomes. *Pharmacological Research* 43, no. 2:161-171.

Lee, W. H., S. Kang, P. P. Vlachos, and Y. W. Lee. 2009. A novel in vitro ischemia/reperfusion injury model. *Archives of Pharmacal Research* 32, no. 3:421-429.

Lee, Y. J., J. H. Lee, and H. J. Han. 2006. Extracellular adenosine triphosphate protects oxidative stress-induced increase of p21(WAF1/Cip1) and p27(Kip1) expression in primary cultured renal proximal tubule cells: role of PI3K and Akt signaling. *Journal of cellular physiology* 209, no. 3:802-810.

Li, A., et al. 2005. Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. *Angiogenesis* 8, no. 1:63-71.

Li, N., and M. Karin. 1999. Is NF-kappaB the sensor of oxidative stress? *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 13, no. 10:1137-1143.

Li, Q., and J. F. Engelhardt. 2006. Interleukin-1beta induction of NFkappaB is partially regulated by H2O2-mediated activation of NFkappaB-inducing kinase. *The Journal of biological chemistry* 281, no. 3:1495-1505.

Lieberthal, W., and S. K. Nigam. 1998. Acute renal failure. I. Relative importance of proximal vs. distal tubular injury. *The American Journal of Physiology* 275, no. 5 Pt 2:F623-31.

Linfert, D., T. Chowdhry, and H. Rabb. 2009. Lymphocytes and ischemia-reperfusion injury. *Transplantation reviews (Orlando, Fla.)* 23, no. 1:1-10.

Liu, J., et al. 2004. Redox regulation of pancreatic cancer cell growth: role of glutathione peroxidase in the suppression of the malignant phenotype. *Human Gene Therapy* 15, no. 3:239-250.

Liu, X., and J. L. Zweier. 2001. A real-time electrochemical technique for measurement of cellular hydrogen peroxide generation and consumption: evaluation in human polymorphonuclear leukocytes. *Free radical biology & medicine* 31, no. 7:894-901.

Loetscher, P., M. Seitz, M. Baggiolini, and B. Moser. 1996. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *The Journal of experimental medicine* 184, no. 2:569-577.

Lubos, E., J. Loscalzo, and D. E. Handy. 2011. Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxidants & redox signaling* 15, no. 7:1957-1997.

Lutz, J., K. Thurmel, and U. Heemann. 2010. Anti-inflammatory treatment strategies for ischemia/reperfusion injury in transplantation. *Journal of inflammation (London, England)* 7, 27.

MacMillan-Crow, L. A., et al. 2001. Mitochondrial tyrosine nitration precedes chronic allograft nephropathy. *Free radical biology & medicine* 31, no. 12:1603-1608.

Magendiramani, V., et al. 2009. S-allylcysteine attenuates renal injury by altering the expressions of iNOS and matrix metallo proteinase-2 during cyclosporine-induced

nephrotoxicity in Wistar rats. *Journal of applied toxicology : JAT* 29, no. 6:522-530.

Martou, Glyka, et al. 2006. Development of an in vitro model for study of the efficacy of ischemic preconditioning in human skeletal muscle against ischemia-reperfusion injury. *Journal of applied physiology* 101, no. 5:1335-1342.

Matsui, M., et al. 1996. Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Developmental biology* 178, no. 1:179-185.

Maulik, N., and D. K. Das. 2008. Emerging potential of thioredoxin and thioredoxin interacting proteins in various disease conditions. *Biochimica et biophysica acta* 1780, no. 11:1368-1382.

McCord, Joe M., and Irwin Fridovich. 1969. Superoxide Dismutase. An enzymic function for erythrocuprein (Hemocuprein). *Journal of Biological Chemistry* 244, no. 22:6049-6055.

McCubrey, J. A., and R. A. Franklin. 2006. Reactive oxygen intermediates and signaling through kinase pathways. *Antioxidants & redox signaling* 8, no. 9-10:1745-1748.

McLaren, A. J., et al. 1999. Delayed graft function: risk factors and the relative effects of early function and acute rejection on long-term survival in cadaveric renal transplantation. *Clinical transplantation* 13, no. 3:266-272.

Mc-Minn editor (Chapter: Abdomen page 367 in Last's anatomy Regional and applied 8th edition RMH ELBS Churchill Livinstone 8th edition1990)

Meier-Kriesche, H. U., J. D. Schold, T. R. Srinivas, and B. Kaplan. 2004. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 4, no. 3:378-383.

Melov, S., et al. 1998. A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase. *Nature genetics* 18, no. 2:159-163.

Miguel, F., A. C. Augusto, and S. A. Gurgueira. 2009. Effect of acute vs chronic H2O2-induced oxidative stress on antioxidant enzyme activities. *Free radical research* 43, no. 4:340-347.

Miranda-Vizuete, A., et al. 2001. Characterization of Sptrx, a novel member of the thioredoxin family specifically expressed in human spermatozoa. *The Journal of biological chemistry* 276, no. 34:31567-31574.

Mitchell, T., et al. 2011. The mitochondria-targeted antioxidant mitoquinone protects against cold storage injury of renal tubular cells and rat kidneys. *The Journal of pharmacology and experimental therapeutics* 336, no. 3:682-692.

Mukaida, N., S. Okamoto, Y. Ishikawa, and K. Matsushima. 1994. Molecular mechanism of interleukin-8 gene expression. *Journal of leukocyte biology* 56, no. 5:554-558.

Munusamy, Shankar, and Lee A. MacMillan-Crow. 2009. Mitochondrial superoxide plays a crucial role in the development of mitochondrial dysfunction during high glucose exposure in rat renal proximal tubular cells. *Free Radical Biology and Medicine* 46, no. 8:1149-1157.

Muselet-Charlier, C., et al. 2007. Enhanced IL-1beta-induced IL-8 production in cystic fibrosis lung epithelial cells is dependent of both mitogen-activated protein kinases and NF-kappaB signaling. *Biochemical and biophysical research communications* 357, no. 2:402-407.

Nakamura, J., E. R. Purvis, and J. A. Swenberg. 2003. Micromolar concentrations of hydrogen peroxide induce oxidative DNA lesions more efficiently than millimolar concentrations in mammalian cells. *Nucleic acids research* 31, no. 6:1790-1795.

Navarro, M. D., et al. 2011. Significance of preimplantation analysis of kidney biopsies from expanded criteria donors in long-term outcome. *Transplantation* 91, no. 4:432-439.

Ng, C. F., F. Q. Schafer, G. R. Buettner, and V. G. Rodgers. 2007. The rate of cellular hydrogen peroxide removal shows dependency on GSH: mathematical insight into in vivo H2O2 and GPx concentrations. *Free radical research* 41, no. 11:1201-1211.

Nordberg, J., and E. S. J. Arnér. 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radical Biology and Medicine* 31, no. 11:1287-1312.

Ohlemiller, K. K., et al. 1999. Targeted deletion of the cytosolic Cu/Zn-superoxide dismutase gene (Sod1) increases susceptibility to noise-induced hearing loss. *Audiology & Neuro-Otology* 4, no. 5:237-246.

Ojo, A. O., et al. 2001. Survival in recipients of marginal cadaveric donor kidneys compared with other recipients and wait-listed transplant candidates. *Journal of the American Society of Nephrology : JASN* 12, no. 3:589-597.

Oliveira, I. C., N. Mukaida, K. Matsushima, and J. Vilcek. 1994. Transcriptional inhibition of the interleukin-8 gene by interferon is mediated by the NF-kappa B site. *Molecular and cellular biology* 14, no. 8:5300-5308.

Olsen, S., et al. 1989. Primary acute renal failure ("acute tubular necrosis") in the transplanted kidney: morphology and pathogenesis. *Medicine* 68, no. 3:173-187.

Patrakka, J., and K. Tryggvason. 2009. New insights into the role of podocytes in proteinuria. *Nature reviews.Nephrology* 5, no. 8:463-468.

Perico, Norberto, Dario Cattaneo, Mohamed H. Sayegh, and Giuseppe Remuzzi. 2004. Delayed graft function in kidney transplantation. *The Lancet* 364, no. 9447:1814-1827.

Perkins, N. D. 2007. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nature reviews.Molecular cell biology* 8, no. 1:49-62.

Pigeolet, E., et al. 1990. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. *Mechanisms of ageing and development* 51, no. 3:283-297.

Powis, G., and W. R. Montfort. 2001. Properties and biological activities of thioredoxins. *Annual Review of Biophysics and Biomolecular Structure* 30, 421-455.

Racusen, L. C., et al. 1997. Cell lines with extended in vitro growth potential from human renal proximal tubule: characterization, response to inducers, and comparison with established cell lines. *The Journal of laboratory and clinical medicine* 129, no. 3:318-329.

Raicevic, S., et al. 2010. Oxidative stress in fetal distress: potential prospects for diagnosis. *Oxidative medicine and cellular longevity* 3, no. 3:214-218.

Raj, D. S., et al. 2004. Advanced glycation end products and oxidative stress are increased in chronic allograft nephropathy. *American Journal of Kidney Diseases : The Official Journal of the National Kidney Foundation* 43, no. 1:154-160.

Reuter, S., et al. 2010. IF/TA-related metabolic changes--proteome analysis of rat renal allografts. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 25, no. 8:2492-2501.

Rice-Evans, C., and R. Burdon. 1993. Free radical-lipid interactions and their pathological consequences. *Progress in lipid research* 32, no. 1:71-110.

Rollins, B. J. 1997. Chemokines. *Blood* 90, no. 3:909-928.

Romiti, N., G. Tramonti, and E. Chieli. 2002. Influence of different chemicals on MDR-1 P-glycoprotein expression and activity in the HK-2 proximal tubular cell line. *Toxicology and applied pharmacology* 183, no. 2:83-91.

Rossi, D., and A. Zlotnik. 2000a. The biology of chemokines and their receptors. *Annual Review of Immunology* 18, 217-242.

Rossi, Devora, and Albert Zlotnik. 2000b. The Biology of Chemokines and their Receptors. *Annual Review of Immunology* 18, no. 1:217-242.

Ruffini, P. A., et al. 2007. Manipulating the chemokine-chemokine receptor network to treat cancer. *Cancer* 109, no. 12:2392-2404.

Rundlof, A. K., M. Carlsten, M. M. Giacobini, and E. S. Arner. 2000. Prominent expression of the selenoprotein thioredoxin reductase in the medullary rays of the rat kidney and thioredoxin reductase mRNA variants differing at the 5' untranslated region. *The Biochemical journal* 347 Pt 3, 661-668.

Ryan, M. J., et al. 1994. HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney international* 45, no. 1:48-57.

Saba, Hamida, Shankar Munusamy, and Lee Macmillan-Crow. 2008. Cold preservation mediated renal injury: involvement of mitochondrial oxidative stress. *Renal failure* 30, no. 2:125-133.

Saitoh, M., et al. 1998. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *The EMBO journal* 17, no. 9:2596-2606.

Salahudeen, A. K. 2004. Cold ischemic injury of transplanted kidneys: new insights from experimental studies. *American journal of physiology.Renal physiology* 287, no. 2:F181-7.

Salahudeen, A. K., H. Huang, P. Patel, and J. K. Jenkins. 2000. Mechanism and prevention of cold storage-induced human renal tubular cell injury. *Transplantation* 70, no. 10:1424-1431.

Salahudeen, A. K., M. Joshi, and J. K. Jenkins. 2001. Apoptosis versus necrosis during cold storage and rewarming of human renal proximal tubular cells. *Transplantation* 72, no. 5:798-804.

Sanchez-Pozos, K., et al. 2010. Polymerized type I collagen reduces chronic cyclosporine nephrotoxicity. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 25, no. 7:2150-2158.

Schreck, R., P. Rieber, and P. A. Baeuerle. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *The EMBO journal* 10, no. 8:2247-2258.

Schwarz, A., et al. 2005. Safety and adequacy of renal transplant protocol biopsies. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 5, no. 8:1992-1996.

Schwarz, A., et al. 2005. Risk factors for chronic allograft nephropathy after renal transplantation: a protocol biopsy study. *Kidney international* 67, no. 1:341-348.

Sehirli, A. O., G. Sener, H. Satiroglu, and G. Ayanoglu-Dulger. 2003. Protective effect of N-acetylcysteine on renal ischemia/reperfusion injury in the rat. *Journal of nephrology* 16, no. 1:75-80.

Shalansky, S. J., G. E. Pate, A. Levin, and J. G. Webb. 2005. N-acetylcysteine for prevention of radiocontrast induced nephrotoxicity: The importance of dose and route of administration. *Heart* 91, no. 8:997-999.

Sheldon, R. A., S. Christen, and D. M. Ferriero. 2008. Genetic and pharmacologic manipulation of oxidative stress after neonatal hypoxia-ischemia. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience* 26, no. 1:87-92.

Sheridan, A. M., et al. 1996. Lipid peroxidation contributes to hydrogen peroxide induced cytotoxicity in renal epithelial cells. *Kidney international* 49, no. 1:88-93.

Shoskes, D. A., and P. F. Halloran. 1996. Delayed graft function in renal transplantation: Etiology, management and long-term significance. *Journal of Urology* 155, no. 6:1831-1840.

Sies, H. 1991. Oxidative stress: from basic research to clinical application. *The American Journal of Medicine* 91, no. 3C:31S-38S.

Simonet, W. S., et al. 1994. Long-term impaired neutrophil migration in mice overexpressing human interleukin-8. *The Journal of clinical investigation* 94, no. 3:1310-1319.

Singh, S., et al. 2011. CXCR1 and CXCR2 silencing modulates CXCL8-dependent endothelial cell proliferation, migration and capillary-like structure formation. *Microvascular research*.

Solez, K, et al. 2008. Banff 07 classification of renal allograft pathology: updates and future directions. *American Journal of Transplantation* 8, 4, 753-760.

Smith, P. K., et al. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* 150, no. 1:76-85.

Somack, R., M. G. Saifer, and L. D. Williams. 1991. Preparation of long-acting superoxide dismutase using high molecular weight polyethylene glycol (41,000-72,000 daltons). *Free radical research communications* 12-13 Pt 2, 553-562.

Spiegel, D. M., P. F. Shanley, and B. A. Molitoris. 1990. Mild ischemia predisposes the S3 segment to gentamicin toxicity. *Kidney international* 38, no. 3:459-464.

St-Pierre, J., J. A. Buckingham, S. J. Roebuck, and M. D. Brand. 2002. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *The Journal of biological chemistry* 277, no. 47:44784-44790.

Strieter, R. M., et al. 1995. Role of C-X-C chemokines as regulators of angiogenesis in lung cancer. *Journal of leukocyte biology* 57, no. 5:752-762.

Stroo, I., et al. 2010. Chemokine expression in renal ischemia/reperfusion injury is most profound during the reparative phase. *International immunology* 22, no. 6:433-442.

Tajkhorshid, E., et al. 2002. Control of the selectivity of the aquaporin water channel family by global orientational tuning. *Science (New York, N.Y.)* 296, no. 5567:525-530.

Takahashi, K., et al. 2008. JNK- and IkappaB-dependent pathways regulate MCP-1 but not adiponectin release from artificially hypertrophied 3T3-L1 adipocytes preloaded with palmitate in vitro. *American journal of physiology.Endocrinology and metabolism* 294, no. 5:E898-909.

Takaya, K., et al. 2003. Involvement of ERK pathway in albumin-induced MCP-1 expression in mouse proximal tubular cells. *American journal of physiology.Renal physiology* 284, no. 5:F1037-45.

Tariq, M., et al. 1999. N-acetylcysteine attenuates cyclosporin-induced nephrotoxicity in rats. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 14, no. 4:923-929.

Tepel, Martin, et al. 2000. Prevention of Radiographic-Contrast-Agent-Induced Reductions in Renal Function by Acetylcysteine. *The New England journal of medicine* 343, no. 3:180-184.

Test, S. T., and S. J. Weiss. 1984. Quantitative and temporal characterization of the extracellular H2O2 pool generated by human neutrophils. *The Journal of biological chemistry* 259, no. 1:399-405.

Thierry, A., et al. 2011. Long-term Impact of Subclinical Inflammation Diagnosed by Protocol Biopsy One Year After Renal Transplantation. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons*.

Tryggvason, K., J. Patrakka, and J. Wartiovaara. 2006. Hereditary proteinuria syndromes and mechanisms of proteinuria. *The New England journal of medicine* 354, no. 13:1387-1401.

Ueno, T., M. Yamada, Y. Igarashi, and T. Ogawa. 2011. N-acetyl cysteine protects osteoblastic function from oxidative stress. *Journal of biomedical materials research.Part A*.

Van Coillie, E., J. Van Damme, and G. Opdenakker. 1999. The MCP/eotaxin subfamily of CC chemokines. *Cytokine & growth factor reviews* 10, no. 1:61-86.

van de Water, B., J. P. Zoeteweij, and J. F. Nagelkerke. 1996. Alkylation-induced oxidative cell injury of renal proximal tubular cells: involvement of glutathione redox-cycle inhibition. *Archives of Biochemistry and Biophysics* 327, no. 1:71-80.

Veraitch, F. S., et al. 2008. The impact of manual processing on the expansion and directed differentiation of embryonic stem cells. *Biotechnology and bioengineering* 99, no. 5:1216-1229.

Verma, I. M., et al. 1995. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes & development* 9, no. 22:2723-2735.

Vesey, D. A., et al. 2009. Isolation and primary culture of human proximal tubule cells. *Methods in molecular biology (Clifton, N.J.)* 466, 19-24.

Vicari, A. P., et al. 1997. TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. *Immunity* 7, no. 2:291-301.

Webb, L., A. Casula, R. Ravanan, and F. Caskey. 2011. Chapter 3: Demographic and Biochemistry Profile of Kidney Transplant Recipients in the UK in 2009: National and Centre-Specific Analyses. *Nephron.Clinical practice* 119 Suppl 2, c53-84.

Weydert, C. J., and J. J. Cullen. 2010. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nature protocols* 5, no. 1:51-66.

Whitehouse, T., et al. 2006. Tissue oxygen and hemodynamics in renal medulla, cortex, and corticomedullary junction during hemorrhage-reperfusion. *American journal of physiology.Renal physiology* 291, no. 3:F647-53.

Whitin, J. C., S. Bhamre, D. M. Tham, and H. J. Cohen. 2002. Extracellular glutathione peroxidase is secreted basolaterally by human renal proximal tubule cells. *American journal of physiology.Renal physiology* 283, no. 1:F20-8.

Wolfe, R. A., et al. 1999. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. *The New England journal of medicine* 341, no. 23:1725-1730.

Wuyts, W. A., et al. 2004. N-acetylcysteine inhibits interleukin-17-induced interleukin-8 production from human airway smooth muscle cells: a possible role for anti-oxidative treatment in chronic lung rejection? *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 23, no. 1:122-127.

Xu, C. F., A. R. Wu, and Y. Z. Shen. 2008. Effects of N-acetylcysteine on mRNA expression of monocyte chemotactic protein and macrophage inflammatory protein 2 in acute necrotizing pancreatitis: experiment with rats. *Zhonghua yi xue za zhi* 88, no. 10:711-715.

Yan, J., et al. 2009. BMP6 attenuates oxidant injury in HK-2 cells via Smad-dependent HO-1 induction. *Free radical biology & medicine* 46, no. 9:1275-1282.

Yarlagadda, S. G., et al. 2009. Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 24, no. 3:1039-1047.

Yoshimura, T., et al. 1987. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proceedings of the National Academy of Sciences of the United States of America* 84, no. 24:9233-9237.

Zelmer, J. L. 2007. The economic burden of end-stage renal disease in Canada. *Kidney international* 72, no. 9:1122-1129.

Zhang, F., S. S. Lau, and T. J. Monks. 2011. The cytoprotective effect of N-acetyl-L-cysteine against ROS-induced cytotoxicity is independent of its ability to enhance glutathione synthesis. *Toxicological sciences : an official journal of the Society of Toxicology* 120, no. 1:87-97.

Zhu, H., L. Zhang, A. R. Amin, and Y. Li. 2008. Coordinated upregulation of a series of endogenous antioxidants and phase 2 enzymes as a novel strategy for protecting renal tubular cells from oxidative and electrophilic stress. *Experimental biology and medicine (Maywood, N.J.)* 233, no. 6:753-765.

Zlotnik, Albert, and Osamu Yoshie. 2000. Chemokines: A New Classification System and Their Role in Immunity. *Immunity* 12, no. 2:121-127.